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NOVEL CARD PROTEINS INVOLVED IN CELL DEATH REGULATION

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the fields of molecular biology and molecular medicine and more specifically to the identification of proteins involved in programmed cell death and associations of these proteins.

BACKGROUND INFORMATION

Programmed cell death is a physiologic process that ensures homeostasis is maintained between cell production and cell turnover in essentially all self-renewing tissues. In many cases, characteristic morphological changes, termed "apoptosis," occur in a dying cell. Since similar changes occur in different types of dying cells, cell death appears to proceed through a common pathway in different cell types.

In addition to maintaining tissue homeostasis, apoptosis also occurs in response to a variety of external stimuli, including growth factor deprivation, alterations in calcium levels, free-radicals, cytotoxic lymphokines, infection by some viruses, radiation and most chemotherapeutic agents. Thus, apoptosis is an inducible event that likely is subject to similar mechanisms of regulation as occur, for example, in a metabolic pathway. In this regard, dysregulation of apoptosis also can occur and is observed, for example, in some types of cancer cells, which survive for a longer

time than corresponding normal cells, and in neurodegenerative diseases where neurons die prematurely. In viral infections, induction of apoptosis can figure prominently in the pathophysiology of the disease process, because immune-based eradication of viral infections depends on elimination of virus-producing host cells by immune cell attack resulting in apoptosis.

Some of the proteins involved in programmed cell death have been identified and associations among some of these proteins have been described. However, additional apoptosis regulating proteins remain to be found and the mechanisms by which these proteins mediate their activity remains to be elucidated. The identification of the proteins involved in cell death and an understanding of the associations between these proteins can provide a means for manipulating the process of apoptosis in a cell and, therefore, selectively regulating the relative lifespan of a cell or its relative resistance to cell death stimuli.

The principal effectors of apoptosis are a family of intracellular proteases known as Caspases, representing an abbreviation for Cysteine Aspartyl Proteases. Caspases are found as inactive zymogens in essentially all animal cells. During apoptosis, the caspases are activated by proteolytic processing at specific aspartic acid residues, resulting in the production of subunits that assemble into an active protease typically consisting of a heterotetramer containing two large and two small subunits (Thornberry and Lazebnik, Science 281:1312-1316 (1998)). The phenomenon of apoptosis is produced directly or indirectly by the activation of caspases in cells, resulting in the proteolytic cleavage of specific substrate proteins. Moreover, in many cases,

caspases can cleave and activate themselves and each other, creating cascades of protease activation and mechanisms for "auto"-activation.

Among the substrates of caspases are the intracellular proforms of cytokines such as pro-Interleukin-1β (pro-IL-1β) and pro-IL-18. When cleaved by caspases, these pro-proteins are converted to the biologically active cytokines which are then liberated from cells, circulating in the body and eliciting inflammatory immune reactions. Thus, caspases can be involved, in some instances, in cytokine activation and responses to infectious agents, as well as inflammatory and autoimmune diseases. Caspases also participate in signal transduction pathways activated by some cytokine receptors, particularly members of the Tumor Necrosis Factor (TNF) family of cytokine receptors which are capable of activating certain caspase zymogens.

Thus, knowledge about the proteins having domains that interact with and regulate caspases is important for devising strategies for manipulating cell life and death in therapeutically useful ways. The identification of such proteins that contain caspase-interacting domains and the elucidation of the proteins with which they interact, therefore, can form the basis for strategies designed to modulate apoptosis, cytokine production, cytokine receptor signaling, and other cellular processes. Thus a need exists to identify proteins that interact with caspases and other apoptosis related proteins. The present invention satisfies this need and provides additional advantages as well.

SUMMARY OF THE INVENTION

In accordance with the present invention, there are provided novel "NB-ARC and CARD"-containing proteins, designated NAC, as well as several isoforms of NAC produced by alternative mRNA splicing. The invention also provides nucleic acid molecules encoding NAC and its isoforms, vectors containing these nucleic acid molecules and host cells containing the vectors. The invention also provides antibodies that can specifically bind to NAC proteins, including alternative isoforms thereof.

The present invention also provides a screening assay useful for identifying agents that can effectively alter the association of NAC with itself or with other proteins. By altering the self-association of NAC or by altering their interactions with other proteins, an effective agent may increase or decrease the level of caspase proteolytic activity or apoptosis in a cell, or it may increase or decrease the levels of NF-κB, cytokine production, or other events.

The invention also provides methods of altering the activity of NAC in a cell, wherein such increased or decreased activity of NAC can modulate the level of apoptosis or other cellular responses. For example, the activity of NAC in a cell can be increased by introducing into the cell and expressing a nucleic acid sequence encoding these proteins. In addition, the activity of NAC in a cell can be decreased by introducing into the cell and expressing a fragment of NAC, or an antisense nucleotide sequence that is complementary to a portion of a nucleic acid molecule encoding the NAC proteins.

The invention also provides methods for using an agent that can specifically bind NAC or a nucleotide sequence that can bind to a nucleic acid molecule encoding NAC to diagnose a pathology that is characterized by an altered level of apoptosis due to an increased or decreased level of NAC in a cell.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the cloning strategy for NAC and Isoforms of NAC. The NB-ARC domain (filled box), leucine-rich repeats (LRR, filled bars), and the CARD domain (labeled box) are depicted. Relevant restriction sites (RI for EcoRI, X for Xho I) are indicated. Positions for forward PCR primers (1F, 2F, and 3F) and reverse primers (1R, 2R, and 3R) which were used for Reverse Transcriptase-Polymerase Chain Reaction cloning of NAC and NAC-isoforms are shown.

Figure 1B shows multiple isoforms of NAC. Isoforms of NAC are generated by alternative mRNA splicing, based on cDNA cloning results. The same symbols as in Figure 1A are used. Two alternatively spliced exons are shown as dotted boxes and hatched boxes, respectively. Note that longer and shorter versions of the CARD domain are produced ($CARD_L$ and $CARD_S$). The four resultant isoforms are described as NACα, NACβ, NACγ and NACδ.

Sub c'

Figure 1C shows the cDNA and amino acid sequence of the longest NAC isoform (also set for in SEQ ID NOs:1 and 2). The nucleotide sequences of the two alternatively spliced exons (nucleotides 2870-2959, and 3784-3915, respectively, and amino acids 918-947 and 1262-1305) are underlined. The positions for the P-loop (Walker A) and Walker B of NB-ARC domain are indicated. The LRR repeats

are in bold letters (amino acids 808-948), and the CARD domain is in bold underlined letters (amino acids 1373-1473).

Figure 1D shows a sequence analysis of NAC: NB-ARC homology. Alignment of the NB-ARC domains of human NAC (amino acids 329-547), CARD4 (amino acids 197-408), and Apaf-1 (amino acids 138-352), and *Caenorhabditis elegans* CED4 (amino acids 154-374). Alignment was conducted using Clustal method (Thompson et al., Nuc. Acids Res. 22:4673-4680 (1994)). Identical and similar residues are shown in black and gray, respectively.

Figure 1E shows alignment of CARD domain of NAC and other CARD-containing proteins. Alignment was conducted using Clustal method. Identical and similar residues are shown in black and gray, respectively.

Figure 2 shows self-association of Long and Short CARD domains of NAC. (A) For *in vitro* binding assays, purified GST fusion proteins immobilized on GSH-sepharose containing $CARD_L$ (lane 3), $CARD_S$ (lane 4), or GST alone (lane 2) were incubated with ^{35}S-labeled, *in vitro* translated $CARD_L$ (top panel), $CARD_S$ (middle panel), or control protein Skp-1 (bottom panel). *In vitro* translation mix (one tenth of input, lane 1) was directly loaded as control. (B) Homophilic interactions of CARD. *In vitro* translated Apaf-1 (-WD) (top panel), CED4 (middle panel), or control Skp-1 (bottom panel) proteins were incubated with GST (lane 2), GST-$CARD_L$ (lane 3), and GST-$CARD_S$(lane 4) immobilized on GSH-sepharose beads. In lane 1, one tenth of input ^{35}S proteins are shown.

Figure 3 shows homophilic interactions of CARD domains detected by yeast two-hybrid method. Yeast cells were

co-transformed with plasmids encoding the indicated proteins fused to LexA DNA binding domain (LexA) and proteins fused to B42 transactivation domain (B42). Transformants were replica-plated on leucine-supplemented plates (Leu+) and leucine-deficient plates (Leu-) to assess protein interactions. β-galactosidase activity (LacZ) was measured for each transformant, and were scaled as: absent (-), weak (+/-), detectable (+), strong (++), very strong (+++), and strongest (++++).

Figure 4 shows self-association of NB-ARC domain of NAC. *In vitro* translated, ^{35}S-labeled rat reticulocyte lysates (1 μl) containing NB-ARC (lanes 2 and 3) or Skp-1 (as a control; lanes 5 and 6) were incubated with purified GST-NB-ARC (lanes 3 and 6) or GST alone (lanes 2 and 5) immobilized on GSH-sepharose beads for *in vitro* binding assays. In lanes 1 and 4, one tenth of input ^{35}S proteins are shown.

Figure 5 shows that NAC forms complexes with Apaf-1 and CED4. (A) Complex formation with human Apaf-1. 293T cells were transiently transfected with an expression plasmid encoding HA-tagged human Apaf-1 lacking the C-terminal WD repeats [HA-Apaf-1 (ΔWD)] in the presence (lanes 2 and 3) or absence (lane 1) of a plasmid encoding myc-tagged full-length NAC (myc-NAC). Transfected cells were lysed and subjected to immunoprecipitation (IP) with either a mouse monoclonal antibody to myc (lanes 1 and 3) or a control mouse IgG (lane 2). Proteins from the immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis (WB) using anti-HA antibodies (bottom panel) followed by anti-myc antibodies (top panel). One tenth of the total cell lysates derived from each transfection were loaded directly in the gel as a control (Lysate). (B) Complex

formation with *C. elegans* CED4 protein. Identical procedures and conditions described for Apaf-1 in (A) were employed for CED4 interaction studies with NAC.

Figure 6 shows that NAC interacts with pro-Casp8, but not pro-Casp9. (A) Interaction with pro-Casp8. 293T cells were transfected with an expression plasmid encoding HA-tagged human pro-Casp8 [HA-Casp8 (C/A)], which harbors an alanine replacement of the catalytic cysteine residue, in the presence (lanes 2 and 3) or absence (lane 1) of myc-NAC expression plasmid. Transfected cells were lysed and subjected to immunoprecipitation (IP) with either anti-myc antibodies (lanes 1 and 3) or a control antibody (lane 2). The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting (WB) for pro-Casp8 (bottom panel) using anti-HA antibodies or for NAC (top panel) using anti-myc antibodies. One tenth of the total cell lysates of each transfection was loaded directly in gels as a control (Lysate). (B) Interaction with pro-Casp9. Identical procedures and conditions described for Casp8 were used for Casp9 interaction studies with NAC. The Casp9 expression plasmid [Flag-Casp9 (C/A)] contains a C-terminal Flag-tagged form of pro-Casp9 harboring an alanine replacement of the catalytic cysteine residue. The immunoblots were probed for Casp9 (bottom panel) using a rabbit anti-Casp9 polyclonal antibody derived against GST-Casp9 fusion proteins.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided "substantially pure" mammalian CARD-containing proteins, designated NAC and CARD-X. As used herein, the term "NAC" refers to a protein that contains both an

NB-ARC domain and a CARD domain (NAC). The invention NAC proteins represent novel members of the "CARD domain" family of proteins, which family includes CED-4 and Apaf-1. An invention NAC comprises a NB-ARC domain and a CARD domain, and optionally further comprises a leucine-rich repeat domain and/or a TIM-Barrel-like domain.

As used herein, the term "CARD domain" refers to a Caspase Recruitment Domain (Hofmann et al., Trends Biochem. Sci. 22:155-156 (1997)). CARD domains have been found in some members of the Caspase family of cell death proteases. Caspases-1, 2, 4, 5, 9, and 11 contain CARD domains near their NH_2-termini. These CARD domains mediate interactions of the zymogen inactive forms of caspases with other proteins which can either activate or inhibit the activation of these enzymes. For example, the CARD domain of pro-caspase-9 binds to the CARD domain of a caspase-activating protein called Apaf-1 (Apoptosis Protease Activating Factor-1). Similarly, the CARD domain of pro-caspase-1 permits interactions with another CARD protein known as Cardiac (also referred to as RIP2 and RICK), which results in activation of the caspase-1 protease (Thome et al., Curr. Biol. 16:885-888 (1998)). And, pro-caspase-2 binds to the CARD protein Raidd (also know as Cradd), which permits recruitment of pro-caspase-2 to Tumor Necrosis Factor (TNF) Receptor complexes and which results in activation of the caspase-2 protease (Ahmad et al., Cancer Res. 57:615-619 (1997)). CARD domains can also participate in homotypic interactions with themselves, resulting in self-association of proteins that contain these protein-interaction domains and producing dimeric or possibly even oligomeric complexes.

CARD domains can be found in association with other types of functional domains within a single polypeptide, thus providing a mechanism for bringing a functional domain into close proximity or contact with a target protein via CARD:CARD associations involving two CARD-containing proteins. For example, the *Caenorhabiditis elegans* cell death gene ced-4 encodes a protein that contains a CARD domain and a ATP-binding oligomerization domain called an NB-ARC domain (van der Biezen and Jones Curr Biol 8:R226-R227). The CARD domain of the CED-4 protein interacts with the CARD domain of a pro-caspase called CED-3. The NB-ARC domain allows CED-4 to self-associate, thereby forming an oligomeric complex which brings associated pro-CED-3 molecules into close proximity to each other. Because most pro-caspases possess at least a small amount of protease activity even in their unprocessed form, the assembly of a complex that brings the proforms of caspase into juxtaposition can result in trans-processing of zymogens, producing the proteolytically processed and active caspase. Thus, CED-4 employs a CARD domain for binding a pro-caspase and an NB-ARC domain for self-oligomerization, resulting in caspase clustering, proteolytic processing and activation.

Numerous CED-4-related proteins have recently been identified. These proteins belong to the CED-4 family of proteins, and include CED-4 (Yuan and Horvitz, Development 116:309-320 (1992)), Apaf-1, (Zou et al., Cell 90:405-413 (1997)), Dark (Rodriguez et al., Nature Cell Biol. 1:272-279 (1999)), and CARD4/Nod1 (Bertin et al., J. Biol. Chem. 274:12955-12958 (1999) and Inohara et al., J. Biol. Chem. 274:14560-14567 (1999)). As used herein, a CED-4 family member is a protein that comprises a NB-ARC domain and a CARD domain.

The CED-4 homolog in humans and rodents, referred to as Apaf-1, has been found to function similarly. The Apaf-1 protein contains a (i) CARD domain, (ii) NB-ARC domain, and (iii) multiple copies of a WD-repeat domain. In contrast to CED-4 which can spontaneously oligomerize, the mammalian Apaf-1 protein is an inactive monomer until induced to oligomerize by binding of a co-factor protein, cytochrome c (Li et al., Cell 91:479-489 (1997)). In Apaf-1, the WD repeat domains prevent oligomerization of the Apaf-1 protein, until coming into contact with cytochrome c. Thus, the WD-repeats function as a negative-regulatory domain that maintains Apaf-1 in a latent state until cytochrome c release from damaged mitochondria triggers the assembly of an oligomeric Apaf-1 complex (Saleh, J. Biol. Chem. 274:17941-17945 (1999)). By binding pro-caspase-9 through its CARD domain, Apaf-1 oligomeric complexes are thought to bring the zymogen forms of caspase-9 into close proximity, permitting them to cleave each other and produce the proteolytic processed and active caspase-9 protease (Zou et al., J. Biol. Chem. 274:11549-11556 (1999)).

In addition to their role in caspase-activation, CARD domains have been implicated in other cellular processes. Some CARD-containing proteins, for example, induce activation of the transcription factor NF-κB. NF-κB activation is induced by many cytokines and plays an important role in cytokine receptor signal transduction mechanisms (DiDonato et al., Nature 388:548-554 (1997)). Moreover, CARD domains are found in some proteins that inhibit rather than activate caspases, such as the IAP (Inhibitor of Apoptosis Protein) family members, cIAP1 and cIAP2 (Rothe et al., Cell 83:1243-1252 (1995)) and oncogenic mutants of the Bcl-10 protein

(Willis et al., Cell 96:35-45 (1999)). Also, though caspase activation resulting from CARD domain interactions is often involved in inducing apoptosis, other caspases are primarily involved in proteolytic processing and activation of inflammatory cytokines (such as pro-IL-1β and pro-IL-18). Thus, CARD-containing proteins can also be involved in cytokine production, thus regulating immune and inflammatory responses.

In view of the function of the CARD domain within invention NAC proteins, invention NAC proteins or CARD-domain containing fragments thereof, are contemplated herein for use in methods to modulate apoptosis, cytokine production, cytokine receptor signaling, and other cellular processes. Invention NAC proteins or CARD-domain containing fragments thereof are also contemplated in methods to identify CARD-binding agents that modulate apoptosis, cytokine production, cytokine receptor signaling, and other cellular processes.

In one embodiment, a CARD domain of an invention NAC comprises a sequence with at least 50% identity to the CARD domain of NAC (see, e.g., residues 1373-1473 of SEQ ID NO:2). More preferably, a CARD domain of the invention comprises a sequence with at least 60% identity to the CARD domain of NAC. Most preferably, a CARD domain of the invention comprises a sequence with at least 75% identity to the CARD domain of NAC. Typically, a CARD domain of the invention comprises a sequence with at least 95% identity to the CARD domain of NAC.

As described herein, invention NAC or CARD-X proteins can associate with other CARD-containing proteins. In particular, the association of the CARD domain of invention NAC proteins with another

CARD-containing protein, such as Apaf-1, CED-4, caspases-1, 2, 9, 11, cIAPs-1 and 2, CARDIAK, Raidd, Dark, CARD4, and other NAC or CARD-X, and the like, is sufficiently specific such that the bound complex can form *in vivo* in a cell or *in vitro* under suitable conditions. Similarly therefore, an invention NAC protein can associate with another NAC protein by CARD:CARD association.

A NAC protein of the invention further can associate with pro-caspases, caspases or with caspase-associated proteins, thereby modulating caspase proteolytic activity. Caspase proteolytic activity is associated with apoptosis of cells, and additionally with cytokine production. Therefore, an invention NAC can modulate apoptosis or cytokine production by modulating caspase proteolytic activity. As used herein a "caspase" is any member of the cysteine aspartyl proteases that associates with a NAC protein of the invention or with a NAC associated protein. Similarly, a "pro-caspase" is an inactive or less-active precursor form of a caspase, which is typically converted to the more active caspase form by a proteolytic event.

CARD-containing proteins are also known to induce activation of the transcription factor NF-κB. Thus, an invention NAC can also modulate transcription by modulation of NF-κB activity.

A NAC protein of the invention also comprises a NB-ARC domain. As described herein, a NB-ARC domain of the invention NAC protein comprises a sequence wherein the identity of residues in either the P-Loop (Walker A) or Walker B regions is at least 60% relative to the residues of NAC (see, e.g., residues 329-343 and 407-412 of SEQ ID NO:2; see Figure 1C). Preferably, an NB-ARC

domain of the invention NAC comprises a sequence wherein the overall identity of residues in the P-Loop (Walker A) and Walker B regions is at least 60% relative to the residues of NAC. More preferably, an NB-ARC domain of the invention comprises a sequence with at least 60% identity to the entire NB-ARC domain of NAC (see, e.g., residues 329-547 of SEQ ID NO:2). Most preferably, an NB-ARC domain of the invention comprises a sequence with at least 80% identity to the entire NB-ARC domain of NAC.

The NB-ARC domain of the invention NAC proteins associates with other proteins, particularly with proteins comprising NB-ARC domains. Thus, a functional NB-ARC domain associates with NB-ARC domain-containing proteins by way of NB-ARC:NB-ARC association. As used herein, the term "associate" or "association" means that NAC can bind to a protein relatively specifically and, therefore, can form a bound complex. In particular, the association of the NB-ARC domain of NAC with another NB-ARC domain-containing proteins is sufficiently specific such that the bound complex can form *in vivo* in a cell or *in vitro* under suitable condition. Further, a NB-ARC domain demonstrates both nucleotide-binding (e.g., ATP-binding) and hydrolysis activities, which is typically required for its ability to associate with NB-ARC domain-containing proteins. Thus, an NB-ARC domain of the invention NAC comprises one or more nucleotide binding sites. As used herein, a nucleotide binding site is a portion of a protein that specifically binds a nucleotide such as, e.g., ATP, and the like. Typically, the nucleotide binding site of NB-ARC will comprise a P-loop, a kinase 2 motif, or a kinase 3a motif of the invention NAC (these motifs are defined, for example, in van der Biezen and Jones, supra). Preferably, the nucleotide binding site of NB-ARC

comprises a P-loop of the invention NAC.

An invention NAC, therefore, is capable of CARD:CARD association and/or NB-ARC:NB-ARC association, resulting in a multifunctional protein capable of one or more specific associations with other proteins. An invention NAC can modulate cell processes such as apoptosis, cytokine production, and the like. For example, it is contemplated herein that an invention NAC protein can increase the level of apoptosis in a cell. It is also contemplated herein that an invention NAC can decrease the level of apoptosis in a cell. For example, a NAC which does not induce apoptosis may form hetero-oligomers with a NAC which is apoptotic, thus interfering with the apoptosis-inducing activity of NAC.

In another embodiment of the invention the NAC protein of the invention also contains Leucine-Rich Repeats (LRR) domain, similar to a LRR described in another CARD protein known as CARD4 (also known as Nod1) (Inohara et al., J. Biol. Chem. 274:14560-14567 (1999)). Unlike CARD-4 (Nod1), however, the CARD domain of NAC is located at the Carboxyl end of the protein whereas the CARD domain of CARD-4 (Nod1) is found at the NH_2-end of the protein. The function of the LRR domain is to mediate specific interactions with other proteins.

As used herein, leucine-rich repeat (LRR) domain of the invention NAC comprises a sequence with at least 50% identity to the LRR domain of NAC (see, e.g., residues 808-948 of SEQ ID NO:2). Preferably, a LRR domain of the invention NAC comprises a sequence with at least 60% identity to the LRR domain of NAC. More preferably, a LRR region of the invention NAC comprises a sequence with at least 75% identity to the LRR domain of NAC. Most preferably, a LRR region of the invention NAC comprises a sequence with at least 95% identity to the LRR domain of

NAC.

It is further contemplated herein that a shortened LRR of the invention NAC may be used. A shortened LRR of the invention comprises a sequence with at least 90% identity to the splice variant form of the LRR (see, e.g., residues 808-917 of SEQ ID NO:2), and does not contain more than 90% of the residues in the splice region (see, e.g., residues 918-947 of SEQ ID NO:2). Preferably, the shortened LRR does not contain more than 70% of the residues in the splice region. More preferably, the shortened LRR does not contain more than 50% of the residues in the splice region. The shortened LRR will be of particular utility when the protein:protein interaction activity of a NAC comprising a shortened LRR differs from that observed for a NAC comprising the full-length LRR. Activity of a NAC with a shortened LRR will be determined by one or more of the assays disclosed herein, and shall be considered to differ from that of a NAC comprising the full-length LRR if any protein:protein interactions are altered by 10% or more, or if caspase activity or apoptotic activity is altered by 10% or more.

In a further embodiment of the invention, invention NAC proteins contain a TIM-Barrel-like domain with similarity to TIM-barrel proteins. TIM-Barrel domains are well known in the art and typically consist of eight alternating α-helices and β-strands forming a barrel-like structure, but may contain 7 α-helices and/or β-strands in some instances. TIM-barrels have been found in some

enzymes, such as aldolase, but also mediate protein interactions in some instances.

As used herein, a TIM-Barrel-like domain of an invention NAC comprises a sequence with at least 50% identity to the TIM-Barrel-like domain of NAC (residues 1079-1320 of SEQ ID NO:2). Preferably, a TIM-barrel-like domain of the invention NAC comprises a sequence with at least 60% identity to the TIM-Barrel-like domain of NAC. More preferably, a TIM-barrel domain of the invention NAC comprises a sequence with at least 70% identity to the TIM-barrel-like domain of NAC. Most preferably, a TIM-barrel-like domain of the invention NAC comprises a sequence with at least 80% identity to the TIM-barrel-like domain of NAC.

Presently preferred NAC proteins of the invention include proteins that comprise substantially the same amino acid sequences as the protein sequence set forth in SEQ ID NOs:2, 4, and 6, as well as biologically active, functional fragments thereof.

Those of skill in the art will recognize that numerous residues of the above-described sequences can be substituted with other, chemically, sterically and/or electronically similar residues without substantially altering the biological activity of the resulting NAC protein species. In addition, larger polypeptide sequences containing substantially the same sequence as amino acids set forth in SEQ ID NOs:2, 4, and 6, therein are contemplated.

As employed herein, the term "substantially the same amino acid sequence" refers to amino acid sequences having at least about 70% identity with respect to the

reference amino acid sequence, and retaining comparable functional and biological activity characteristic of the protein defined by the reference amino acid sequence. Preferably, proteins having "substantially the same amino acid sequence" will have at least about 80%, more preferably 90% amino acid identity with respect to the reference amino acid sequence; with greater than about 95% amino acid sequence identity being especially preferred. It is recognized, however, that polypeptides (or nucleic acids referred to hereinbefore) containing less than the described levels of sequence identity arising as splice variants or that are modified by conservative amino acid substitutions, or by substitution of degenerate codons are also encompassed within the scope of the present invention.

The term "biologically active" or "functional", when used herein as a modifier of invention NACs, or polypeptide fragments thereof, refers to a polypeptide that exhibits functional characteristics similar to a NAC. Biological activities of NAC are, for example, the ability to bind, preferably *in vivo*, to a CARD-containing protein or a NB-ARC-containing protein, or to homo-oligomerize, or to modulate protease activation, particularly caspase activation, or to modulate NF-κB activity, or to modulate apoptosis, as described herein. Such NAC binding activity can be assayed, for example, using the methods described herein. Another biological activity of NAC is the ability to act as an immunogen for the production of polyclonal and monoclonal antibodies that bind specifically to an invention NAC. Thus, an invention nucleic acid encoding NAC will encode a polypeptide specifically recognized by an antibody that also specifically recognizes a NAC protein (preferably human) including the amino acid set forth in SEQ ID

NOs:2, 4, 6, 10 or 12. Such immunologic activity may be assayed by any method known to those of skill in the art. For example, a test-polypeptide encoded by a NAC cDNA can be used to produce antibodies, which are then assayed for their ability to bind to an invention NAC protein including the sequence set forth in SEQ ID NOs:2, 4, 6, 10 or 12. If the antibody binds to the test-polypeptide and the protein including the sequence encoded by SEQ ID NOs:2, 4, 6, 10 or 12 with substantially the same affinity, then the polypeptide possesses the requisite immunologic biological activity.

As used herein, the term "substantially purified" means a protein that is in a form that is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally associated with a protein in a cell. A substantially purified NAC can be obtained by a variety of methods well-known in the art, e.g., recombinant expression systems described herein, precipitation, gel filtration, ion-exchange, reverse-phase and affinity chromatography, and the like. Other well-known methods are described in Deutscher et al., Guide to Protein Purification: Methods in Enzymology Vol. 182, (Academic Press, (1990)), which is incorporated herein by reference. Alternatively, the isolated polypeptides of the present invention can be obtained using well-known recombinant methods as described, for example, in Sambrook et al., supra., (1989).

In addition to the ability of invention NAC proteins, or fragements thereof, to interact with other, heterologous proteins (i.e., NB-ARC and CARD-containing proteins), invention NAC and CARD-X proteins have the ability to self-associate. This self-association is

possible through interactions between CARD domains, and also through interactions between NB-ARC domains. Further, self-association can take place as a result of interactions between LRR and TIM-Barrel-like domains.

In accordance with the invention, there are also provided mutations and fragments of NAC which have activity different than a wild type NAC activity. As used herein, a "mutation" can be any deletion, insertion, or change of one or more amino acids in the wild type protein sequence, and a "fragment" is any truncated form, either carboxy-terminal, amino-terminal, or both, of the wild type protein. Preferably, the different activity of the mutation or fragment is a result of the mutant protein or fragment maintaining some but not all of the activities of wild type NAC. For example, a fragment of NAC can contain a CARD domain and LRR and TIM-Barrel-like domains, but lack a functional NB-ARC domain. Such a fragment will maintain a portion of the wild type NAC activity (e.g., CARD domain functionality), but not all wild type activities (e.g., lacking an active NB-ARC domain). The resultant fragment will therefore have activity different than wild type NAC activity. In one embodiment, the activity of the fragment will be "dominant negative." A dominant negative activity will allow the fragment to reduce or inactivate the activity of one or more isoforms of wild type NAC.

sub c2 Isoforms of the NAC proteins are also provided ~~which~~ arise from alternative mRNA splicing ~~and~~ may alter or modify the interactions ~~of~~ the NAC protein with other proteins. For ~~example~~, three novel isoforms of NAC are provided ~~herein~~ and designated: NACβ, NACγ and NACδ (set ~~forth~~ as SEQ ID Nos:1, 3 and 5, respectively). The amino acid sequence and the portion of cDNA encoding the amino

acid sequence of NACβ is shown in Figure 1C, and the NACβ cDNA and amino acid sequences are listed as SEQ ID NOs: 1 and 2, respectively. NACβ represents the NAC splice variant in which both splice regions are present in the translated polypeptide, thereby including the nucleic acids 1-4422 of the NAC cDNA sequence and amino acids 1-1473 of the NAC protein sequence of Figure 1C. NACγ represents the NAC splice variant in which neither splice region is present in the translated polypeptide, thereby including the nucleic acids 1-2869, 2960-3783, and 3916-4422 of the NAC cDNA sequence and amino acids 1-917, 948-1261, and 1306-1473 of the NAC protein sequence of Figure 1C. The NACγ cDNA and amino acid sequences are listed as SEQ ID NOs:3 and 4, respectively. NACδ represents the NAC splice variant in which only the more carboxy-terminal splice region is present in the translated polypeptide, thereby including the nucleic acids 1-2869, and 2960-4422 of the NAC cDNA sequence and amino acids 1-917, and 948-1473 of the NAC protein sequence of Figure 1C. The NACδ cDNA and amino acid sequences are listed as SEQ ID NOs:5 and 6, respectively.

In another embodiment of the invention, chimeric proteins are provided comprising NAC, or a functional fragment thereof, fused with another protein or functional fragment thereof. Functional fragments of NAC include, for example, NB-ARC, CARD, LRR and TIM-Barrel-like domains, as defined herein. Proteins with which the NAC or functional fragment thereof are fused will include, for example, glutathione-S-transferase, an antibody, or other proteins or functional fragments thereof which facilitate recovery of the chimera. Further proteins with which the NAC or functional fragment thereof are fused will include, for example,

luciferase, green fluorescent protein, an antibody, or other proteins or functional fragments thereof which facilitate identification of the chimera. Still further proteins with which the NAC or functional fragment thereof are fused will include, for example, the LexA DNA binding domain, ricin, α-sarcin, an antibody, or other proteins which have therapeutic properties or other biological activity.

Further invention chimeric proteins contemplated herein are chimeric proteins wherein a domain of the NAC is replaced by a similar such domain from a heterologous protein. For example, the NB-ARC domain of NAC, as described above, can be replaced by the NB-ARC domain of Apaf-1, and the like. Another example of such a chimera is a protein wherein the CARD domain of NAC is replaced by the CARD domain from CED-4, and the like.

The CARD-X protein contains a CARD domain and a region with similarity to TIM-Barrel-like domains, but otherwise is distinct from NAC. The cDNA sequence encoding CARD-X (SEQ ID NO:7) reveals that it arises from a separate gene from NAC. The predicted CARD-X amino acid sequence (SEQ ID NO:8), in particular, does not contain an NB-ARC domain.

A CARD domain of the CARD-X protein comprises a sequence with at least 50% identity to the CARD domain of CARD-X (residues 343-431 of SEQ ID NO:8). More preferably, a CARD domain of the invention comprises a sequence with at least 60% identity to the CARD domain of CARD-X. Most preferably, a CARD domain of the invention comprises a sequence with at least 75% identity to the CARD domain of CARD-X. Typically, a CARD domain of the

invention comprises a sequence with at least 95% identity to the CARD domain of CARD-X.

A TIM-Barrel-like domain of CARD-X comprises a sequence with at least 50% identity to the TIM-Barrel domain of CARD-X (residues 56-331 of SEQ ID NO:8). Preferably, a TIM-barrel domain of the invention NAC comprises a sequence with at least 60% identity to the TIM-Barrel domain of CARD-X. More preferably, a TIM-barrel domain of the invention CARD-X comprises a sequence with at least 70% identity to the TIM-barrel domain of CARD-X. Most preferably, a TIM-barrel domain of the CARD-X comprises a sequence with at least 80% identity to the TIM-barrel domain of CARD-X.

In one embodiment, invention chimeric CARD-containing proteins provided herein are designated NAC-X. Nucleic acids that encode NAC-X are also provided herein. Alternative isoforms of the NAC-X proteins and the corresponding nucleic acids that encode the alternative isoforms are also provided. As used herein, the term "NAC-X" refers to chimeric proteins comprising portions of a NAC and portions of CARD-X. For example, one type of NAC-X protein is a NACδ-X, wherein a portion of NACδ, for example, the TIM-Barrel-like domain of NACδ, is replaced by a portion of CARD-X, for example, the TIM-Barrel-like domain of CARD-X. It is within the scope of this invention that a protein comprising portions of a domain common to both NAC and CARD-X, particularly the CARD and TIM-Barrel-like domains, can comprise a chimera of NAC and CARD-X. For example, a NACβ-X protein can have residues 1-1397 from SEQ ID NO:2 immediately followed by residues 364-402 from SEQ ID NO:8, which are in turn immediately followed by residues 1436-1473 from SEQ ID NO:2, thus forming a chimeric CARD domain.

In one embodiment, a NAC-X protein will comprise an NB-ARC domain of NAC, as previously described, and the CARD domain of CARD-X. In another embodiment, a NAC-X protein will comprise the NB-ARC domain and LRR domain of NAC, the CARD domain of CARD-X, and the TIM-Barrel-like domain from either NAC or CARD-X or a chimera from both. In yet another embodiment, NAC-X will comprise the NB-ARC and LRR domains of NAC and the CARD and TIM-Barrel-like domains of CARD-X. For example, invention chimeric proteins can include residues between 1-947 and 1-1078 of NACβ (SEQ ID NO:2) or between 1-918 and 1-1048 of NACγ or NACδ (SEQ ID NOs:4 and 6, respectively) in chimera with residues between 1-431 and 56-431 of CARD-X (SEQ ID NO:8). A particular invention chimera is termed NAC-X1 a protein, and comprises the following sequences: NACβ-X1, residues 1-1078 of NACβ and residues 56-431 of CARD-X, having the resultant amino acid sequence listed in SEQ ID NO:10; NACγ/δ-X1 residues 1-1048 of NACγ or NACδ and residues 56-431 of CARD-X, having the resultant amino acid sequence listed in SEQ ID NO:12. The cDNA encoding NACβ-X1 comprises cDNA residues 1-3234 of NACβ and 166-1293 of CARD-X, having the resultant sequence listed in SEQ ID NO:9; and the cDNA encoding NACγ/δ-X1 proteins comprise cDNA residues 1-3144 of NACγ or NACδ and 166-1293 of CARD-X, having the resultant sequence listed in SEQ ID NO:11.

Another embodiment of the invention provides NAC, or a functional fragment thereof, fused with a moiety to form a conjugate. As used herein, a "moiety" can be a physical, chemical or biological entity which contributes

functionality to NAC or a functional fragment thereof. Functionalities contributed by a moiety include therapeutic or other biological activity, or the ability to facilitate identification or recovery of NAC. Therefore, a moiety will include molecules known in the art to be useful for detection of the conjugate by, for example, by fluorescence, magnetic imaging, detection of radioactive emission. A moiety may also be useful for recovery of the conjugate, for example a His tag or other known tags used for protein isolation/purification, or a physical substance such as a bead. A moiety can be a therapeutic compound, for example, a cytotoxic drug which can be useful to effect a biological change in cells to which the conjugate localizes.

An example of the means for preparing the invention polypeptide(s) is to express nucleic acids encoding the NAC in a suitable host cell, such as a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), or a mammalian cell, using methods well known in the art, and recovering the expressed polypeptide, again using well-known methods. Invention polypeptides can be isolated directly from cells that have been transformed with expression vectors as described below herein. The invention polypeptide, biologically functional fragments, and functional equivalents thereof can also be produced by chemical synthesis. For example, synthetic polypeptides can be produced using Applied Biosystems, Inc. Model 430A or 431A automatic peptide synthesizer (Foster City, CA) employing the chemistry provided by the manufacturer.

Also encompassed by the term NAC are functional fragments or polypeptide analogs thereof. The term "functional fragment" refers to a peptide fragment that

is a portion of a full length NAC protein, provided that the portion has one or more biological activities, as defined above, that is characteristic of the corresponding full length NAC. For example, a functional fragment of an invention NAC protein can have one or more of the protein:protein binding activities prevalent in NAC. In addition, the characteristic of a functional fragment of invention NAC proteins to elicit an immune response is useful for obtaining an anti-NAC antibodies. Thus, the invention also provides functional fragments of invention NAC proteins, which can be identified using the binding and routine methods, such as bioassays described herein.

The term "polypeptide analog" includes any polypeptide having an amino acid residue sequence substantially the same as a sequence specifically shown herein in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the ability to functionally mimic an NAC as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The amino acid length of functional fragments or polypeptide analogs of the present invention can range from about 5 amino acids up to the full-length protein

sequence of an invention NAC. In certain embodiments, the amino acid lengths include, for example, at least about 10 amino acids, at least about 20, at least about 30, at least about 40, at least about 50, at least about 75, at least about 100, at least about 150, at least about 200, at least about 250 or more amino acids in length up to the full-length NAC protein sequence.

As used herein the phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue, provided that such polypeptide displays the required binding activity. The phrase "chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions

of residues, relative to the sequence of a polypeptide whose sequence is shown herein, so long as the required activity is maintained.

The present invention also provides compositions containing an acceptable carrier and any of an isolated, purified NAC mature protein or functional polypeptide fragments thereof, alone or in combination with each other. These polypeptides or proteins can be recombinantly-derived, chemically synthesized or purified from native sources. As used herein, the term "acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as phosphate buffered saline solution, water and emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The NAC compositions described herein can be used, for example, in methods described hereinafter.

In accordance with another embodiment of the invention, substantially pure nucleic acid molecules, and functional fragments thereof, are provided, which encode invention NACs. Exemplary invention nucleic acid molecules are those comprising substantially the same nucleotide sequence encoding NACβ (SEQ ID NO: 1), NACγ (SEQ ID NO: 3), and NACδ (SEQ ID NO: 5).

The nucleic acid molecules described herein are useful for producing invention proteins, when such nucleic acids are incorporated into a variety of protein expression systems known to those of skill in the art. In addition, such nucleic acid molecules or fragments thereof can be labeled with a readily detectable substituent and used as hybridization probes for assaying for the presence and/or amount of an invention NAC gene or mRNA transcript in a given sample. The nucleic acid

molecules described herein, and fragments thereof, are also useful as primers and/or templates in a PCR reaction for amplifying genes encoding invention proteins described herein.

The term "nucleic acid" (also referred to as polynucleotides) encompasses ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), probes, oligonucleotides, and primers. DNA can be either complementary DNA (cDNA) or genomic DNA, e.g. a gene encoding a NAC. One means of isolating a nucleic acid encoding an NAC polypeptide is to probe a mammalian genomic library with a natural or artificially designed DNA probe using methods well known in the art. DNA probes derived from the NAC gene are particularly useful for this purpose. DNA and cDNA molecules that encode NAC polypeptides can be used to obtain complementary genomic DNA, cDNA or RNA from mammalian (e.g., human, mouse, rat, rabbit, pig, and the like), or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic libraries, by methods described in more detail below. Such nucleic acids may include, but are not limited to, nucleic acids comprising substantially the same nucleotide sequence as set forth in SEQ ID NOs:1 (NACβ), 3 (NACγ), and 5 (NACδ).

Use of the terms "isolated" and/or "purified" and/or "substantially purified" in the present specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been produced in such form by the hand of man, and thus are separated from their native *in vivo* cellular environment, and are substantially free of any other species of nucleic acid or protein. As a result of this human intervention, the recombinant DNAs,

RNAs, polypeptides and proteins of the invention are useful in ways described herein that the DNAs, RNAs, polypeptides or proteins as they naturally occur are not.

Invention NAC proteins and nucleic acids encoding such, can be obtained from any species of organism, such as prokaryotes, eukaryotes, plants, fungi, vertebrates, invertebrates, and the like. A particular species can be mammalian, As used herein, "mammalian" refers to a subset of species from which an invention NAC is derived, e.g., human, rat, mouse, rabbit, monkey, baboon, bovine, porcine, ovine, canine, feline, and the like. A preferred NAC herein, is human NAC.

In one embodiment of the present invention, cDNAs encoding the invention NACs disclosed herein comprise substantially the same nucleotide sequence as the coding region set forth in any of SEQ ID NOs :1, 3 and 5. Preferred cDNA molecules encoding the invention proteins comprise the same nucleotide sequence as the coding region set forth in any of SEQ ID NOs :1, 3 and 5.

As employed herein, the term "substantially the same nucleotide sequence" refers to DNA having sufficient identity to the reference polynucleotide, such that it will hybridize to the reference nucleotide under moderately stringent hybridization conditions. In one embodiment, DNA having substantially the same nucleotide sequence as the reference nucleotide sequence encodes substantially the same amino acid sequence as that set forth in any of SEQ ID NOs:2, 4, 6, 10 or 12. In another embodiment, DNA having "substantially the same nucleotide sequence" as the reference nucleotide sequence has at least 60% identity with respect to the reference nucleotide sequence. DNA having at least 70%, more

preferably at least 90%, yet more preferably at least 95%, identity to the reference nucleotide sequence is preferred.

This invention also encompasses nucleic acids which differ from the nucleic acids shown in SEQ ID NOs :1, 3 and 5, but which have the same phenotype. Phenotypically similar nucleic acids are also referred to as "functionally equivalent nucleic acids". As used herein, the phrase "functionally equivalent nucleic acids" encompasses nucleic acids characterized by slight and non-consequential sequence variations that will function in substantially the same manner to produce the same protein product(s) as the nucleic acids disclosed herein. In particular, functionally equivalent nucleic acids encode polypeptides that are the same as those encoded by the nucleic acids disclosed herein or that have conservative amino acid variations. For example, conservative variations include substitution of a non-polar residue with another non-polar residue, or substitution of a charged residue with a similarly charged residue. These variations include those recognized by skilled artisans as those that do not substantially alter the tertiary structure of the protein.

Further provided are nucleic acids encoding NAC polypeptides that, by virtue of the degeneracy of the genetic code, do not necessarily hybridize to the invention nucleic acids under specified hybridization conditions. Preferred nucleic acids encoding the invention NACs are comprised of nucleotides that encode substantially the same amino acid sequence as set forth in SEQ ID NOs:2, 4, 6, 10 or 12.

Thus, an exemplary nucleic acid encoding an invention NAC may be selected from:

(a) DNA encoding the amino acid sequence set forth in SEQ ID NOs:2, 4, 6, 10 or 12,

(b) DNA that hybridizes to the DNA of (a) under moderately stringent conditions, wherein said DNA encodes biologically active NAC, or

(c) DNA degenerate with respect to (b) wherein said DNA encodes biologically active NAC.

Hybridization refers to the binding of complementary strands of nucleic acid (i.e., sense:antisense strands or probe:target-DNA) to each other through hydrogen bonds, similar to the bonds that naturally occur in chromosomal DNA. Stringency levels used to hybridize a given probe with target-DNA can be readily varied by those of skill in the art.

The phrase "stringent hybridization" is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions.

As used herein, the phrase "moderately stringent hybridization" refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, more preferably about 85% identity to the target DNA; with

greater than about 90% identity to target-DNA being especially preferred. Preferably, moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C.

The phrase "high stringency hybridization" refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (i.e., if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C.

The phrase "low stringency hybridization" refers to conditions equivalent to hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 42°C, followed by washing in 1X SSPE, 0.2% SDS, at 50°C. Denhart's solution and SSPE (see, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) are well known to those of skill in the art as are other suitable hybridization buffers.

As used herein, the term "degenerate" refers to codons that differ in at least one nucleotide from a reference nucleic acid, e.g., SEQ ID NOs :1, 3 and 5, but encode the same amino acids as the reference nucleic acid. For example, codons specified by the triplets "UCU", "UCC", "UCA", and "UCG" are degenerate with

respect to each other since all four of these codons encode the amino acid serine.

Preferred nucleic acids encoding the invention polypeptide(s) hybridize under moderately stringent, preferably high stringency, conditions to substantially the entire sequence, or substantial portions (i.e., typically at least 15-30 nucleotides) of the nucleic acid sequence set forth in SEQ ID NOs :1, 3 and 5.

The invention nucleic acids can be produced by a variety of methods well-known in the art, e.g., the methods described herein, employing PCR amplification using oligonucleotide primers from various regions of SEQ ID NOs :1, 3 and 5, and the like.

In accordance with a further embodiment of the present invention, optionally labeled NAC-encoding cDNAs, or fragments thereof, can be employed to probe library(ies) (e.g., cDNA, genomic, and the like) for additional nucleic acid sequences encoding novel NACs. Construction of suitable mammalian cDNA libraries, including mammalian cDNA libraries, is well-known in the art. Screening of such a cDNA library is initially carried out under low-stringency conditions, which comprise a temperature of less than about 42°C, a formamide concentration of less than about 50%, and a moderate to low salt concentration.

Presently preferred probe-based screening conditions comprise a temperature of about 37°C, a formamide concentration of about 20%, and a salt concentration of about 5X standard saline citrate (SSC; 20X SSC contains 3M sodium chloride, 0.3M sodium citrate, pH 7.0). Such conditions will allow the identification of sequences

which have a substantial degree of similarity with the probe sequence, without requiring perfect homology. The phrase "substantial similarity" refers to sequences which share at least 50% homology. Preferably, hybridization conditions will be selected which allow the identification of sequences having at least 70% homology with the probe, while discriminating against sequences which have a lower degree of homology with the probe. As a result, nucleic acids having substantially the same nucleotide sequence as SEQ ID NOs :1, 3 and 5 are obtained.

As used herein, a nucleic acid "probe" is single-stranded DNA or RNA, or analogs thereof, that has a sequence of nucleotides that includes at least 15, at least 20, at least 50, at least 100, at least 200, at least 300, at least 400, or at least 500 contiguous bases that are the same as (or the complement of) any contiguous bases set forth in any of SEQ ID NOs :1, 3 and 5. Preferred regions from which to construct probes include 5' and/or 3' coding regions of SEQ ID NOs :1, 3 and 5. In addition, the entire cDNA encoding region of an invention NAC, or the entire sequence corresponding to SEQ ID NOs :1, 3 and 5, may be used as a probe. Probes may be labeled by methods well-known in the art, as described hereinafter, and used in various diagnostic kits.

As used herein, the terms "label" and "indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal. Any label or indicating means can be linked to invention nucleic acid probes, expressed proteins, polypeptide fragments, or antibody molecules. These

atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry.

The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturation to form a fluorochrome (dye) that is a useful immunofluorescent tracer. A description of immunofluorescent analytic techniques is found in DeLuca, "Immunofluorescence Analysis", in Antibody As a Tool, Marchalonis et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which is incorporated herein by reference.

In one embodiment, the indicating group is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, and the like. In another embodiment, radioactive elements are employed labeling agents. The linking of a label to a substrate, i.e., labeling of nucleic acid probes, antibodies, polypeptides, and proteins, is well known in the art. For instance, an invention antibody can be labeled by metabolic incorporation of radiolabeled amino acids provided in the culture medium. See, for example, Galfre et al., Meth. Enzymol., 73:3-46 (1981). Conventional means of protein conjugation or coupling by activated functional groups are particularly applicable. See, for example, Aurameas et al., Scand. J. Immunol., Vol. 8, Suppl. 7:7-23 (1978), Rodwell et al., Biotech., 3:889-894 (1984), and U.S. Patent No. 4,493,795.

Also provided are antisense-nucleic acids having a sequence capable of binding specifically with full-length or any portion of an mRNA that encodes NAC polypeptides so as to prevent translation of the mRNA. The

antisense-nucleic acid may have a sequence capable of binding specifically with any portion of the sequence of the cDNA encoding NAC polypeptides. As used herein, the phrase "binding specifically" encompasses the ability of a nucleic acid sequence to recognize a complementary nucleic acid sequence and to form double-helical segments therewith via the formation of hydrogen bonds between the complementary base pairs. An example of an antisense-nucleic acid is an antisense-nucleic acid comprising chemical analogs of nucleotides.

Compositions comprising an amount of the antisense-nucleic acid, described above, effective to reduce expression of NAC polypeptides by passing through a cell membrane and binding specifically with mRNA encoding NAC polypeptides so as to prevent translation and an acceptable hydrophobic carrier capable of passing through a cell membrane are also provided herein. Suitable hydrophobic carriers are described, for example, in U.S. Patent Nos. 5,334,761; 4,889,953; 4,897,355, and the like. The acceptable hydrophobic carrier capable of passing through cell membranes may also comprise a structure which binds to a receptor specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind to a cell-type specific receptor.

Antisense-nucleic acid compositions are useful to inhibit translation of mRNA encoding invention polypeptides. Synthetic oligonucleotides, or other antisense chemical structures are designed to bind to mRNA encoding NAC polypeptides and inhibit translation of mRNA and are useful as compositions to inhibit expression of NAC associated genes in a tissue sample or in a subject.

In accordance with another embodiment of the invention, kits are provided for detecting mutations, duplications, deletions, rearrangements and aneuploidies in NAC genes comprising at least one invention probe or antisense nucleotide.

The present invention provides means to modulate levels of expression of NAC polypeptides by employing synthetic antisense-nucleic acid compositions (hereinafter SANC) which inhibit translation of mRNA encoding these polypeptides. Synthetic oligonucleotides, or other antisense-nucleic acid chemical structures designed to recognize and selectively bind to mRNA, are constructed to be complementary to full-length or portions of an NAC coding strand, including nucleotide sequences set forth in SEQ ID NOs :1, 3 and 5 . The SANC is designed to be stable in the blood stream for administration to a subject by injection, or in laboratory cell culture conditions. The SANC is designed to be capable of passing through the cell membrane in order to enter the cytoplasm of the cell by virtue of physical and chemical properties of the SANC which render it capable of passing through cell membranes, for example, by designing small, hydrophobic SANC chemical structures, or by virtue of specific transport systems in the cell which recognize and transport the SANC into the cell. In addition, the SANC can be designed for administration only to certain selected cell populations by targeting the SANC to be recognized by specific cellular uptake mechanisms which bind and take up the SANC only within select cell populations. In a particular embodiment the SANC is an antisense oligonucleotide.

For example, the SANC may be designed to bind to a receptor found only in a certain cell type, as discussed *supra*. The SANC is also designed to recognize and selectively bind to target mRNA sequence, which may correspond to a sequence contained within the sequences shown in SEQ ID NOs :1, 3 and 5. The SANC is designed to inactivate target mRNA sequence by either binding thereto and inducing degradation of the mRNA by, for example, RNase I digestion, or inhibiting translation of mRNA target sequence by interfering with the binding of translation-regulating factors or ribosomes, or inclusion of other chemical structures, such as ribozyme sequences or reactive chemical groups which either degrade or chemically modify the target mRNA. SANCs have been shown to be capable of such properties when directed against mRNA targets (see Cohen et al., TIPS, 10:435 (1989) and Weintraub, Sci. American, January (1990), pp.40; both incorporated herein by reference).

In accordance with yet another embodiment of the present invention, there is provided a method for the recombinant production of invention NAC by expressing the above-described nucleic acid sequences in suitable host cells. Recombinant DNA expression systems that are suitable to produce NAC described herein are well-known in the art. For example, the above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof.

Suitable expression vectors are well-known in the art, and include vectors capable of expressing DNA operatively linked to a regulatory sequence, such as a

promoter region that is capable of regulating expression of such DNA. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the inserted DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

Prokaryotic transformation vectors are well-known in the art and include pBlueskript and phage Lambda ZAP vectors (Stratagene, La Jolla, CA), and the like. Other suitable vectors and promoters are disclosed in detail in U.S. Patent No. 4,798,885, issued January 17, 1989, the disclosure of which is incorporated herein by reference in its entirety.

Other suitable vectors for transformation of *E. coli* cells include the pET expression vectors (Novagen, see U.S. patent 4,952,496), e.g., pET11a, which contains the T7 promoter, T7 terminator, the inducible *E. coli* lac operator, and the lac repressor gene; and pET 12a-c, which contain the T7 promoter, T7 terminator, and the *E. coli* ompT secretion signal. Another suitable vector is the pIN-IIIompA2 (see Duffaud et al., Meth. in Enzymology, 153:492-507, 1987), which contains the lpp promoter, the lacUV5 promoter operator, the ompA secretion signal, and the lac repressor gene.

In accordance with another embodiment of the present invention, there are provided "recombinant cells" containing the nucleic acid molecules (i.e., DNA or mRNA) of the present invention. Methods of transforming

suitable host cells, preferably bacterial cells, and more preferably *E. coli* cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1989).

Exemplary methods of transformation include, e.g., transformation employing plasmids, viral, or bacterial phage vectors, transfection, electroporation, lipofection, and the like. The heterologous DNA can optionally include sequences which allow for its extrachromosomal maintenance, or said heterologous DNA can be caused to integrate into the genome of the host (as an alternative means to ensure stable maintenance in the host).

Host organisms contemplated for use in the practice of the present invention include those organisms in which recombinant production of heterologous proteins has been carried out. Examples of such host organisms include bacteria (e.g., *E. coli*), yeast (e.g., *Saccharomyces cerevisiae, Candida tropicalis, Hansenula polymorpha* and *P. pastoris*; see, e.g., U.S. Patent Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), mammalian cells (e.g., HEK293, CHO and Ltk$^-$ cells), insect cells, and the like. Presently preferred host organisms are bacteria. The most preferred bacteria is *E. coli*.

In one embodiment, nucleic acids encoding the invention NAC can be delivered into mammalian cells, either *in vivo* or *in vitro* using suitable viral vectors well-known in the art. Suitable retroviral vectors, designed specifically for "gene therapy" methods, are

described, for example, in WIPO publications WO 9205266 and WO 9214829, which provide a description of methods for efficiently introducing nucleic acids into human cells. In addition, where it is desirable to limit or reduce the *in vivo* expression of the invention NAC, the introduction of the antisense strand of the invention nucleic acid is contemplated.

For example, in one embodiment of the present invention, adenovirus-transferrin/polylysine-DNA (TfAdpl-DNA) vector complexes (Wagner et al., Proc. Natl. Acad. Sci., USA, 89:6099-6103 (1992); Curiel et al., Hum. Gene Ther., 3:147-154 (1992); Gao et al., Hum. Gene Ther., 4:14-24 (1993)) are employed to transduce mammalian cells with heterologous NAC nucleic acid. Any of the plasmid expression vectors described herein may be employed in a TfAdpl-DNA complex.

In accordance with yet another embodiment of the present invention, there are provided anti-NAC antibodies having specific reactivity with an NAC polypeptides of the present invention. The present invention also provides anti-NACβ, anti-NACγ, anti-NACδ, anti-NACβ-X1, or anti-NACγ/δ-X1 antibodies. It should be recognized that an antibody of the invention can be specific for an epitope that is present only in a particular type of NAC or can be specific for an epitope that is common to more than one type of NAC. For example, an anti-NACδ antibody can be specific for only NACδ or for more than one member of the NAC family. As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as polypeptide fragments of antibodies that retain a specific binding activity for a specific antigen of at least about 1 x 105 M-1. One

skilled in the art would know that, for example, anti-NACβ antibody fragments or anti-NACγ antibody fragments such as Fab, F(ab')2, Fv and Fd fragments can retain specific binding activity for a NACβ or a NACγ, respectively, and, thus, are included within the definition of an antibody. In addition, the term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies and fragments of antibodies that retain binding activity. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science 246:1275-1281 (1989), which is incorporated herein by reference.

Invention antibodies can be produced by methods known in the art using invention polypeptides, proteins or portions thereof as antigens. For example, polyclonal and monoclonal antibodies can be produced by methods well known in the art, as described, for example, in Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory (1988)), which is incorporated herein by reference. Invention polypeptides can be used as immunogens in generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding polypeptide. Altered antibodies such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical

synthesis or recombinant methods described, for example, in Sambrook et al., supra., and Harlow and Lane, supra. Both anti-peptide and anti-fusion protein antibodies can be used. (see, for example, Bahouth et al., Trends Pharmacol. Sci. 12:338 (1991); Ausubel et al., Current Protocols in Molecular Biology (John Wiley and Sons, NY (1989) which are incorporated herein by reference).

In the case of monoclonal antibodies specific to NAC, it is also contemplated herein that the invention includes hybridomas and any other type of cell line which produces a monoclonal antibody. Methods of preparing hybridomas are described for example, in Sambrook et al., supra., and Harlow and Lane, supra; and preparation of any non-hybridoma cell line producing a monoclonal antibody specific to NAC can be carried out in accordance with the methods known in the art and methods described herein for protein expression in cells such as bacterial cells, yeast cells, amphibian cells, mammalian cells, and the like.

Antibody so produced can be used, inter alia, in diagnostic methods and systems to detect the level of NAC present in a mammalian, preferably human, body sample, such as tissue or vascular fluid. Such antibodies can also be used for the immunoaffinity or affinity chromatography purification of the invention NAC. In addition, methods are contemplated herein for detecting the presence of an invention NAC protein in a tissue or cell, comprising contacting the cell with an antibody that specifically binds to NAC polypeptides, under conditions permitting binding of the antibody to the NAC polypeptides, detecting the presence of the antibody bound to the NAC polypeptide, and thereby detecting the presence of invention polypeptides. With respect to the

detection of such polypeptides, the antibodies can be used for *in vitro* diagnostic or *in vivo* imaging methods.

Immunological procedures useful for *in vitro* detection of target NAC polypeptides in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionucleotides, enzymes, fluorogens, chromogens and chemiluminescent labels.

Invention anti-NAC antibodies are contemplated for use herein to modulate the activity of the NAC polypeptide in living animals, in humans, or in biological tissues or fluids isolated therefrom. The term "modulate" refers to a compound's ability to increase (e.g., via an agonist) or inhibit (e.g., via an antagonist) the biological activity of an invention NAC protein, such as the capablity of binding CARD-containing proteins, NB-ARC-containing proteins, to modulate the activity of proteases such as caspases, to modulate the activity of NF-κB, and to modulate apoptosis. Accordingly, compositions comprising a carrier and an amount of an antibody having specificity for NAC polypeptides effective to inhibit naturally occurring ligands or NAPs from binding to invention NAC polypeptides are contemplated herein. For example, a monoclonal antibody directed to an epitope of an invention NAC polypeptide including an amino acid

sequence set forth in SEQ ID NOs:2, 4, 6, 10 or 12, can be useful for this purpose.

The present invention further provides transgenic non-human mammals that are capable of expressing exogenous nucleic acids encoding NAC polypeptides. As employed herein, the phrase "exogenous nucleic acid" refers to nucleic acid sequence which is not native to the host, or which is present in the host in other than its native environment (e.g., as part of a genetically engineered DNA construct). In addition to naturally occurring levels of NAC, invention NAC can either be overexpressed or underexpressed (such as in the well-known knock-out transgenics) in transgenic mammals.

Also provided are transgenic non-human mammals capable of expressing nucleic acids encoding NAC polypeptides so mutated as to be incapable of normal activity, i.e., do not express native NAC. The present invention also provides transgenic non-human mammals having a genome comprising antisense nucleic acids complementary to nucleic acids encoding NAC polypeptides, placed so as to be transcribed into antisense mRNA complementary to mRNA encoding NAC polypeptides, which hybridizes to the mRNA and, thereby, reduces the translation thereof. The nucleic acid may additionally comprise an inducible promoter and/or tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of nucleic acids are DNA or cDNA having a coding sequence substantially the same as the coding sequence shown in SEQ ID NOs :1, 3 or 5. An example of a non-human transgenic mammal is a transgenic mouse. Examples of

tissue specificity-determining elements are the metallothionein promoter and the L7 promoter.

Animal model systems which elucidate the physiological and behavioral roles of NAC polypeptides are also provided, and are produced by creating transgenic animals in which the expression of the NAC polypeptide is altered using a variety of techniques. Examples of such techniques include the insertion of normal or mutant versions of nucleic acids encoding an NAC polypeptide by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos to produce a transgenic animal. (See, for example, Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual (Cold Spring Harbor Laboratory, (1986)).

Also contemplated herein, is the use of homologous recombination of mutant or normal versions of NAC genes with the native gene locus in transgenic animals, to alter the regulation of expression or the structure of NAC polypeptides (see, Capecchi et al., Science 244:1288 (1989); Zimmer et al., Nature 338:150 (1989); which are incorporated herein by reference). Homologous recombination techniques are well known in the art. Homologous recombination replaces the native (endogenous) gene with a recombinant or mutated gene to produce an animal that cannot express native (endogenous) protein but can express, for example, a mutated protein which results in altered expression of NAC polypeptides.

In contrast to homologous recombination, microinjection adds genes to the host genome, without removing host genes. Microinjection can produce a transgenic animal that is capable of expressing both

endogenous and exogenous NAC. Inducible promoters can be linked to the coding region of nucleic acids to provide a means to regulate expression of the transgene. Tissue specific regulatory elements can be linked to the coding region to permit tissue-specific expression of the transgene. Transgenic animal model systems are useful for *in vivo* screening of compounds for identification of specific ligands, i.e., agonists and antagonists, which activate or inhibit NAC protein responses.

A further embodiment of the invention provides a method to identify agents that can effectively alter NAC activity, for example the ability of NAC to association with one or more heterologous proteins. Thus, the present invention provides a screening assay useful for identifying an effective agent, which can alter the association of a NAC with a NAC associated protein, such as a CARD-containing protein and/or an NB-ARC-containing protein. Since CARD-containing proteins and NB-ARC-containing proteins are involved in apoptosis, the identification of such effective agents can be useful for modulating the level of apoptosis in a cell in a subject having a pathology characterized by an increased or decreased level of apoptosis.

Further, since invention NAC proteins comprise CARD domains, effective agents can be useful for modulation of any other CARD domain activity. These additional CARD domain activities include, for example, NF-κB activity modulation, cytokine receptor signal transduction, and caspase activation/inhibition, regardless of whether the effected caspase is involved in apoptosis or some alternative cellular process such as proteolytic processing and activation of inflammatory cytokines.

As used herein, the term "agent" means a chemical or biological molecule such as a simple or complex organic molecule, a peptide, a peptido-mimetic, a protein or an oligonucleotide that has the potential for altering the association of NAC with a heterologous protein or altering the ability of NAC to self-associate or altering the nucleotide binding and/or hydrolysis activity of NAC. In addition, the term "effective agent" is used herein to mean an agent that can, in fact, alter the association of NAC with a heterologous protein or altering the ability of NAC to self-associate or altering the nucleotide binding and/or hydrolysis activity of NAC. For example, an effective agent may be an anti-NAC antibody or a NAC-associated-protein.

As used herein, the term "alter the association" means that the association between two specifically interacting proteins either is increased or is decreased due to the presence of an effective agent. As a result of an altered association of NAC with another protein in a cell, the activity of the NAC or the NAC associated protein can be increased or decreased, thereby modulating a biological process, for example, the level of apoptosis in the cell. As used herein, the term "alter the activity" means that the agent can increase or decrease the activity of a NAC in a cell, thereby modulating a biological process in a cell, for example, the level of apoptosis in the cell. For example, an effective agent can increase or decrease the NB-ARC:NB-ARC-associating activity of a NAC, without affecting the association of the NAC with a CARD-containing protein. Modulation of the ATP hydrolysis activity can modulate the ability of NAC proteins to associate with other NB-ARC-containing proteins, such as Apaf-1, thereby modulating any process effected by such association between NAC and an

NB-ARC-containing protein. Similarly, the term "alters the association" of NAC with another protein refers to increasing or decreasing, or otherwise changing the association between a NAC and a protein that specifically binds to NAC (i.e., a NAC associated protein).

An effective agent can act by interfering with the ability of a NAC to associate with another protein, or can act by causing the dissociation of NAC from a complex with a NAC-associated protein, wherein the ratio of bound NAC to free NAC is related to the level of a biological process, for example, apoptosis, in a cell. For example, binding of a ligand to a NAC-associated protein can allow the NAC-associated protein, in turn, to bind a NAC. The association, for example, of a CARD-containing protein and a NAC can result in activation or inhibition of the NB-ARC:NB-ARC-associating activity of NAC. In the presence of an effective agent, the association of a NAC and a CARD-containing protein can be altered, which can thereby alter the activation of caspases in the cell. As a result of the altered caspase activation, the level of apoptosis in a cell can be increased or decreased. Thus, the identification of an effective agent that alters the association of NAC with another protein can allow for the use of the effective agent to increase or decrease the level of apoptosis in a cell.

An effective agent can be useful, for example, to increase the level of apoptosis in a cell such as a cancer cell, which is characterized by having a decreased level of apoptosis as compared to its normal cell counterpart. An effective agent also can be useful, for example, to decrease the level of apoptosis in a cell such as a T lymphocyte in a subject having a viral disease such as acquired immunodeficiency syndrome, which

is characterized by an increased level of apoptosis in an infected T cell as compared to a normal T cell. Thus, an effective agent can be useful as a medicament for altering the level of apoptosis in a subject having a pathology characterized by increased or decreased apoptosis. In addition, an effective agent can be used, for example, to decrease the level of apoptosis and, therefore, increase the survival time of a cell such as a hybridoma cell in culture. The use of an effective agent to prolong the survival of a cell *in vitro* can significantly improve bioproduction yields in industrial tissue culture applications.

A NAC that lacks the ability to bind the NB-ARC domain of another protein but retains the ability to self-associate via its CARD domain or to bind to other CARD-containing proteins is an example of an effective agent, since the expression of a non-NB-ARC-associating NAC in a cell can alter the association of a the endogenous NAC protein with itself or with NAC associated proteins.

Thus, it should be recognized that a mutation of a NAC can be an effective agent, depending, for example, on the normal level of NAC/NAC-associated protein that occurs in a particular cell type. In addition, an active fragment of a NAC can be an effective agent, provided the active fragment can alter the association of NAC and another protein in a cell. Such active fragments, which can be peptides as small as about five amino acids, can be identified, for example, by screening a peptide library (see, for example, Ladner et al., U.S. Patent No: 5,223,409, which is incorporated herein by reference) to identify peptides that can bind a NAC-associated protein.

Similarly, a peptide or polypeptide portion of a NAC-associated protein also can be an effective agent. A peptide such as the C-terminal peptide of NAC-associated protein can be useful, for example, for decreasing the association of NAC with a CARD-containing protein or a NB-ARC-containing protein in a cell by competing for binding to the NAC. A non-naturally occurring peptido-mimetic also can be useful as an effective agent. Such a peptido-mimetic can include, for example, a peptoid, which is peptide-like sequence containing N-substituted glycines, or an oligocarbamate. A peptido-mimetic can be particularly useful as an effective agent due, for example, to having an increased stability to enzymatic degradation *in vivo*.

A screening assay to identify an effective agent can be performed *in vivo* using the two hybrid system or can be performed *in vitro* as disclosed herein. The yeast two hybrid system, for example, can be used to screen a panel of agents to identify effective agents that alter the association of NAC with another protein. An effective agent can be identified by detecting an altered level of transcription of a reporter gene. For example, the level of transcription of a reporter gene due to the bridging of a DNA-binding domain and trans-activation domain by a NAP and NAC hybrids can be determined in the absence and in the presence of an agent. An effective agent, which alters the association between NAC and another protein, can be identified by a proportionately altered level of transcription of the reporter gene as compared to the control level of transcription in the absence of the agent.

As understood by those of skill in the art, assay methods for identifying agents that modulate NAC activity

generally require comparison to a control. For example, one type of a "control" is a cell or culture that is treated substantially the same as the test cell or test culture exposed to the agent, with the distinction that the "control" cell or culture is not exposed to the agent. Another type of "control" cell or culture may be a cell or culture that is identical to the transfected cells, with the exception that the "control" cell or culture do not express native proteins. Accordingly, the response of the transfected cell to agent is compared to the response (or lack thereof) of the "control" cell or culture to the same agent under the same reaction conditions. Similarly, a "control" can be the extract, partially purified or not, of a cell not exposed to the agent or not expressing certain native proteins. A "control" may also be an isolated compound, for example, a protein (e.g., Skp-1 as used in Examples), which is known to not specifically associate with NAC proteins.

Accordingly, in accordance with another embodiment of the present invention, there is provided a method of identifying an effective agent that alters the association of a NB-ARC and CARD-containing protein (NAC) with a NAC associated protein (NAP), by the steps of:

a. contacting said NAC and NAP proteins, under conditions that allow the NAC and NAP proteins to associate, with an agent suspected of being able to alter the association of the NAC and NAP proteins; and

b. detecting the altered association of the NAC and NAP proteins, wherein the altered association identifies an effective agent.

Methods well-known in the art for detecting the altered association of the NAC and NAP proteins, for example, measuring protein:protein binding, protein degradation or apoptotic activity can be employed in bioassays described herein to identify agents as agonists or antagonists of NAC proteins. As described herein, NAC proteins have the ability to self-associate. Thus, methods for identifying effective agents that alter the association of a NAC protein NAP will also be useful for identifying effective agents that alter the ability of NAC to self-associate. Similarly, CARD-X proteins have the ability to interact with other CARD-containing proteins and to self-associate. Thus, methods for identifying effective agents that alter the association of a NAC and another protein will also be useful for identifying effective agents that alter the ability of CARD-X to self-associate or to associate with a heterologous CARD-containing protein.

As used herein, "conditions that allow said NAC and NAP proteins to associate" refers to environmental conditions in which NAC:NAP specifically associate. Such conditions will typically be aqueous conditions, with a pH between 3.0 and 11.0, and temperature below 100°C. Preferably, the conditions will be aqueous conditions with salt concentrations below the equivalent of 1 M NaCl, and pH between 5.0 and 9.0, and temperatures between 0°C and 50°C. Most preferably, the conditions will range from physiological conditions of normal yeast or mammalian cells, or conditions favorable for carrying out in vitro assays such as immunoprecipitation and GST-NAC:NAP association assays, and the like.

In yet another embodiment of the present invention, there are provided methods for modulating the caspase

modulating activity mediated by NAC proteins, the method comprising:

contacting an NAC protein with an effective, modulating amount of an agonist or antagonist identified by the above-described bioassays.

The present invention also provides *in vitro* screening assays. Such screening assays are particularly useful in that they can be automated, which allows for high through-put screening, for example, of randomly or rationally designed agents such as drugs, peptidomimetics or peptides in order to identify those agents that effectively alter the association of NAC and NAP proteins or the activity of a NAC and, thereby, modulate apoptosis. An *in vitro* screening assay can utilize, for example, a NAC or a NAC fusion protein such as a NAC-glutathione-S-transferase fusion protein (GST/NAC; see Examples). For use in the *in vitro* screening assay, the NAC or NAC fusion protein should have an affinity for a solid substrate as well as the ability to associate with a NAC-associated protein. For example, when a NAC is used in the assay, the solid substrate can contain a covalently attached anti-NAC antibody. Alternatively, a GST/NAC fusion protein can be used in the assay and the solid substrate can contain covalently attached glutathione, which is bound by the GST component of the GST/NAC fusion protein. Similarly, a NAC-associated protein, or a GST/CARD-containing protein or GST/NB-ARC-containing protein fusion protein can be used in an *in vitro* assay as described herein.

An *in vitro* screening assay can be performed by allowing a NAC or NAC-fusion protein, for example, to bind to the solid support, then adding a NAC-associated protein and an agent to be tested. Control reactions,

which do not contain an agent, can be performed in parallel. Following incubation under suitable conditions, which include, for example, an appropriate buffer concentration and pH and time and temperature that permit binding of the particular NAC and NAC-associated protein, the amount of protein that has associated in the absence of an agent and in the presence of an agent can be determined. The association of a NAC-associated protein with a NAC protein can be detected, for example, by attaching a detectable moiety such as a radionuclide or a fluorescent label to a NAC-associated protein and measuring the amount of label that is associated with the solid support, wherein the amount of label detected indicates the amount of association of the NAC-associated protein with a NAC protein. An effective agent is determined by comparing the amount of specific binding in the presence of an agent as compared to the control level of binding, wherein an effective agent alters the association of NAC with the NAC-assocated protein. Such an assay is particularly useful for screening a panel of agents such as a peptide library in order to detect an effective agent.

The invention further provides methods for introducing a nucleic acid encoding a NAC into a cell in a subject, for example, for gene therapy. Viruses are specialized infectious agents that can elude host defense mechanisms and can infect and propagate in specific cell types. Viral based systems provide the advantage of being able to introduce relatively high levels of the heterologous nucleic acid into a variety of cells. Suitable viral vectors for introducing invention nucleic acid encoding an NAC protein into mammalian cells (e.g., vascular tissue segments) are well known in the art. These viral vectors include, for example, Herpes simplex

virus vectors (e.g., Geller et al., Science, 241:1667-1669 (1988)), Vaccinia virus vectors (e.g., Piccini et al., Meth. in Enzymology, 153:545-563 (1987); Cytomegalovirus vectors (Mocarski et al., in Viral Vectors, Y. Gluzman and S.H. Hughes, Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988, pp. 78-84), Moloney murine leukemia virus vectors (Danos et al., Proc. Natl. Acad. Sci., USA, 85:6469 (1980)), adenovirus vectors (e.g., Logan et al., Proc. Natl. Acad. Sci., USA, 81:3655-3659 (1984); Jones et al., Cell, 17:683-689 (1979); Berkner, Biotechniques, 6:616-626 (1988); Cotten et al., Proc. Natl. Acad. Sci., USA, 89:6094-6098 (1992); Graham et al., Meth. Mol. Biol., 7:109-127 (1991)), adeno-associated virus vectors, retrovirus vectors (see, e.g., U.S. Patent 4,405,712 and 4,650,764), and the like. Especially preferred viral vectors are the adenovirus and retroviral vectors.

Suitable retroviral vectors for use herein are described, for example, in U.S. Patent 5,252,479, and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, incorporated herein by reference, which provide a description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, the mouse mammary tumor virus vectors (e.g., Shackleford et al., Proc. Natl. Acad. Sci. USA, 85:9655-9659 (1988)), and the like.

In particular, the specificity of viral vectors for particular cell types can be utilized to target predetermined cell types. Thus, the selection of a viral vector will depend, in part, on the cell type to be targeted. For example, if a neurodegenerative disease is to be treated by increasing the level of a NAC in

neuronal cells affected by the disease, then a viral vector that targets neuronal cells can be used. A vector derived from a herpes simplex virus is an example of a viral vector that targets neuronal cells (Battleman et al., J. Neurosci. 13:941-951 (1993), which is incorporated herein by reference). Similarly, if a disease or pathological condition of the hematopoietic system is to be treated, then a viral vector that is specific for a particular blood cell or its precursor cell can be used. A vector based on a human immunodeficiency virus is an example of such a viral vector (Carroll et al., J. Cell. Biochem. 17E:241 (1993), which is incorporated herein by reference). In addition, a viral vector or other vector can be constructed to express a nucleic acid encoding a NAC in a tissue specific manner by incorporating a tissue-specific promoter or enhancer into the vector (Dai et al., Proc. Natl. Acad. Sci. USA 89:10892-10895 (1992), which is incorporated herein by reference).

For gene therapy, a vector containing a nucleic acid encoding a NAC or an antisense nucleotide sequence can be administered to a subject by various methods. For example, if viral vectors are used, administration can take advantage of the target specificity of the vectors. In such cases, there in no need to administer the vector locally at the diseased site. However, local administration can be a particularly effective method of administering a nucleic acid encoding a NAC. In addition, administration can be via intravenous or subcutaneous injection into the subject. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection. Injection of viral vectors into the spinal fluid also can be an effective mode of

administration, for example, in treating a neurodegenerative disease.

Receptor-mediated DNA delivery approaches also can be used to deliver a nucleic acid molecule encoding a NAC into cells in a tissue-specific manner using a tissue-specific ligand or an antibody that is non-covalently complexed with the nucleic acid molecule via a bridging molecule (Curiel et al., Hum. Gene Ther. 3:147-154 (1992); Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987), each of which is incorporated herein by reference). Direct injection of a naked or a nucleic acid molecule encapsulated, for example, in cationic liposomes also can be used for stable gene transfer into non-dividing or dividing cells *in vivo* (Ulmer et al., Science 259:1745-1748 (1993), which is incorporated herein by reference). In addition, a nucleic acid molecule encoding a NAC can be transferred into a variety of tissues using the particle bombardment method (Williams et al., Proc. Natl. Acad. Sci. USA 88:2726-2730 (1991), which is incorporated herein by reference). Such nucleic acid molecules can be linked to the appropriate nucleotide sequences required for transcription and translation.

A particularly useful mode of administration of a nucleic acid encoding a NAC is by direct inoculation locally at the site of the disease or pathological condition. Local administration can be advantageous because there is no dilution effect and, therefore, the likelihood that a majority of the targeted cells will be contacted with the nucleic acid molecule is increased. Thus, local inoculation can alleviate the targeting requirement necessary with other forms of administration and, if desired, a vector that infects all cell types in

the inoculated area can be used. If expression is desired in only a specific subset of cells within the inoculated area, then a promotor, an enhancer or other expression element specific for the desired subset of cells can be linked to the nucleic acid molecule. Vectors containing such nucleic acid molecules and regulatory elements can be viral vectors, viral genomes, plasmids, phagemids and the like. Transfection vehicles such as liposomes also can be used to introduce a non-viral vector into recipient cells. Such vehicles are well known in the art.

The present invention also provides therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention, such as pharmaceutical compositions, contain a physiologically compatible carrier together with an invention NAC (or functional fragment thereof), a NAC modulating agent, such as a compound (agonist or antagonist) identified by the methods described herein, or an anti-NAC antibody, as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically compatible" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset, and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well known in the art. Typically such compositions are prepared as injectables either as liquid solutions or suspensions; however, solid forms suitable for solution, or suspension, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, as well as combinations of any two or more thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and the like, which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable nontoxic salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid, and the like.

Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium hydroxide, ammonium hydroxide, potassium hydroxide, and the like; and organic bases such as mono-, di-, and tri-alkyl and -aryl amines (e.g., triethylamine, diisopropyl amine, methyl amine, dimethyl amine, and the like) and optionally substituted ethanolamines (e.g., ethanolamine, diethanolamine, and the like).

Physiologically tolerable carriers are well known in the art. Exemplary liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary additional liquid phases include glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

As described herein, an "effective amount" is a predetermined amount calculated to achieve the desired therapeutic effect, e.g., to modulate the protein degradation activity of an invention NAC protein. The required dosage will vary with the particular treatment and with the duration of desired treatment; however, it is anticipated that dosages between about 10 micrograms and about 1 milligram per kilogram of body weight per day will be used for therapeutic treatment. It may be

particularly advantageous to administer such compounds in depot or long-lasting form as discussed hereinafter. A therapeutically effective amount is typically an amount of an NAC-modulating agent or compound identified herein that, when administered in a physiologically acceptable composition, is sufficient to achieve a plasma concentration of from about 0.1 µg/ml to about 100 µg/ml, preferably from about 1.0 µg/ml to about 50 µg/ml, more preferably at least about 2 µg/ml and usually 5 to 10 µg/ml. Therapeutic invention anti-NAC antibodies can be administered in proportionately appropriate amounts in accordance with known practices in this art.

Also provided herein are methods of treating pathologies, said method comprising administering an effective amount of an invention therapeutic composition. Such compositions are typically administered in a physiologically compatible composition.

Exemplary diseases related to abnormal cell proliferation contemplated herein for treatment according to the present invention include cancer pathologies, keratinocyte hyperplasia, neoplasia, keloid, benign prostatic hypertrophy, inflammatory hyperplasia, fibrosis, smooth muscle cell proliferation in arteries following balloon angioplasty (restenosis), and the like. Exemplary cancer pathologies contemplated herein for treatment include, gliomas, carcinomas, adenocarcinomas, sarcomas, melanomas, hamartomas, leukemias, lymphomas, and the like.

Methods of treating pathologies of abnormal cell proliferation will include methods of modulating the activity of one or more oncogenic proteins, wherein the oncogenic proteins specifically interact with NAC.

Methods of modulating the activity of such oncogenic proteins will include contacting the oncogenic protein with a substantially pure NAC or an active fragment (i.e., oncogenic protein-binding fragment) thereof. This contacting will modulate the activity of the oncogenic protein, thereby providing a method of treating a pathology caused by the oncogenic protein. Further methods of modulating the activity of oncogenic proteins will include contacting the oncogenic protein with an agent, wherein the agent modulates the interactions between NAC and the oncogenic protein.

Also contemplated herein, are therapeutic methods using invention pharmaceutical compositions for the treatment of pathological disorders in which there is too little cell division, such as, for example, bone marrow aplasias, immunodeficiencies due to a decreased number of lymphocytes, and the like. Methods of treating a variety of inflammatory diseases with invention therapeutic compositions are also contemplated herein, such as treatment of sepsis, fibrosis (e.g., scarring), arthritis, graft versus host disease, and the like.

The present invention also provides methods for diagnosing a pathology that is characterized by an increased or decreased level of apoptosis in a cell to determine whether the increased or decreased level of apoptosis is due, for example, to increased or decreased expression of a NAC in the cell or to expression of a variant NAC. The identification of such a pathology, which can be due to altered association of a NAC with a NAC-associated protein in a cell, can allow for intervention therapy using an effective agent or a nucleic acid molecule or an antisense nucleotide sequence as described above. In general, a test sample can be

obtained from a subject having a pathology characterized by having or suspected of having increased or decreased apoptosis and can be compared to a control sample from a normal subject to determine whether a cell in the test sample has, for example, increased or decreased expression of NAC. The level of a NAC in a cell can be determined by contacting a sample with a reagent such as an anti-NAC antibody or a NAC-associated protein, either of which can specifically bind a NAC. For example, the level of a NAC in a cell can determined by well known immunoassay or immunohistochemical methods using an anti-NAC antibody (see, for example, Reed et al., supra, 1992; see, also, Harlow and Lane, supra, (1988)). As used herein, the term "reagent" means a chemical or biological molecule that can specifically bind to a NAC or to a bound NAC/NAC-associated protein complex. For example, either an anti-NAC antibody or a NAC-associated protein can be a reagent for a NAC, whereas either an anti-NAC antibody or an anti-NAC-associated protein antibody can be a reagent for a NAC/NAC-associated protein complex.

As used herein, the term "test sample" means a cell or tissue specimen that is obtained from a subject and is to be examined for expression of a NAC in a cell in the sample. A test sample can be obtained, for example, during surgery or by needle biopsy and can be examined using the methods described herein to diagnose a pathology characterized by increased or decreased apoptosis. Increased or decreased expression of a NAC in a cell in a test sample can be determined by comparison to an expected normal level for a NAC in a particular cell type. A normal range of NAC levels in various cell types can be determined by sampling a statistically significant number of normal subjects. In addition, a

control sample can be evaluated in parallel with a test sample in order to determine whether a pathology characterized by increased or decreased apoptosis is due to increased or decreased expression of a NAC. The test sample can be examined using, for example, immunohistochemical methods as described above or the sample can be further processed and examined. For example, an extract of a test sample can be prepared and examined to determine whether a NAC that is expressed in a cell in the sample can associate with a NAC-associated protein in the same manner as a NAC from a control cell or whether, instead, a variant NAC is expressed in the cell.

In accordance with another embodiment of the present invention, there are provided diagnostic systems, preferably in kit form, comprising at least one invention nucleic acid encoding NAC, NAC protein, and/or anti-NAC antibody described herein, in a suitable packaging material. In one embodiment, for example, the diagnostic nucleic acids are derived from any of SEQ ID NOs :1, 3 and 5. Invention diagnostic systems are useful for assaying for the presence or absence of nucleic acid encoding NAC in either genomic DNA or in transcribed nucleic acid (such as mRNA or cDNA) encoding NAC.

A suitable diagnostic system includes at least one invention NAC nucleic acid, NAC protein, and/or anti-NAC antibody, preferably two or more invention nucleic acids, proteins and/or antibodies, as a separately packaged chemical reagent(s) in an amount sufficient for at least one assay. Instructions for use of the packaged reagent are also typically included. Those of skill in the art can readily incorporate invention nucleic probes and/or primers into kit form in combination with appropriate

buffers and solutions for the practice of the invention methods as described herein.

As employed herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit, such as invention nucleic acid probes or primers, and the like. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. The packaging material has a label which indicates that the invention nucleic acids can be used for detecting a particular sequence encoding NAC including the nucleotide sequences set forth in SEQ ID NOs :1, 3 and 5 or mutations or deletions therein, thereby diagnosing the presence of, or a predisposition for, cancer. In addition, the packaging material contains instructions indicating how the materials within the kit are employed both to detect a particular sequence and diagnose the presence of, or a predisposition for, cancer.

The packaging materials employed herein in relation to diagnostic systems are those customarily utilized in nucleic acid-based diagnostic systems. As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding within fixed limits an isolated nucleic acid, oligonucleotide, or primer of the present invention. Thus, for example, a package can be a glass vial used to contain milligram quantities of a contemplated nucleic acid, oligonucleotide or primer, or it can be a microtiter plate well to which microgram quantities of a contemplated nucleic acid probe have been operatively affixed.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter, such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions, and the like.

A diagnostic assay should include a simple method for detecting the amount of a NAC in a sample that is bound to the reagent. Detection can be performed by labeling the reagent and detecting the presence of the label using well known methods (see, for example, Harlow and Lane, supra, 1988; chap. 9, for labeling an antibody). A reagent can be labeled with various detectable moieties including a radiolabel, an enzyme, biotin or a fluorochrome. Materials for labeling the reagent can be included in the diagnostic kit or can be purchased separately from a commercial source. Following contact of a labeled reagent with a test sample and, if desired, a control sample, specifically bound reagent can be identified by detecting the particular moiety.

A labeled antibody that can specifically bind the reagent also can be used to identify specific binding of an unlabeled reagent. For example, if the reagent is an anti-NAC antibody, a second antibody can be used to detect specific binding of the anti-NAC antibody. A second antibody generally will be specific for the particular class of the first antibody. For example, if an anti-NAC antibody is of the IgG class, a second antibody will be an anti-IgG antibody. Such second antibodies are readily available from commercial sources. The second antibody can be labeled using a detectable moiety as described above. When a sample is labeled

using a second antibody, the sample is first contacted with a first antibody, then the sample is contacted with the labeled second antibody, which specifically binds to the first antibody and results in a labeled sample.

In accordance with another embodiment of the invention, a method is provided to identify NAC-associated proteins. As used herein, the term "NAC-associated protein" or "NAP" means a protein that can specifically bind to NAC or its alternative isoforms. Because NAC proteins are known to self-associate, NAC proteins are encompassed by the term NAP. An exemplary NAP is a protein or a polypeptide portion of a protein that can bind the NB-ARC, CARD, LRR, or TIM-Barrel-like domains of NAC. Similarly, the term "CARD-X Associated Protein" or "CAP" refers to a protein that can bind specifically to the CARD-X protein. Likewise, since CARD-X proteins are known to self-associate, CARD-X proteins are encompassed by the term CAP. A NAP or CAP can be identified, for example, using *in vitro* protein binding assays similar to those described in the Examples, by Yeast Two-Hybrid assays similar to those described in the Examples, or by other types of protein-interaction assays and methods.

Using NAC or CARD-X, it is clear to one skilled in the art of protein purification, protein interaction cloning, or protein mass-spectrometry, that NAPs or CAPs can be identified using the methods disclosed herein.

Although the term "NAP" or "CAP" is used generally, it should be recognized that a NAP or CAP that is identified using an assay described herein can be a portion of a protein, which is considered to be a candidate NAP or CAP. As used herein, the term "active

fragment" of a NAP or CAP refers to a protein that corresponds to a polypeptide sequence that can bind NAC or CARD-X, respectively, but that consists of only a portion of the full length protein. Although such polypeptides are considered NAPs or CAPs, it is well known that a cDNA sequence obtained from a cDNA library may not encode the full length protein. Thus, a cDNA can encode a polypeptide that is only a portion of a full length protein but, nevertheless, assumes an appropriate conformation and contains a sufficient region so as to bind NAC or CARD-X. However, in the full length protein, the polypeptide can assume a conformation that does not bind NAC or CARD-X, due for example to steric blocking of the NAP or CAP binding site. Such a full length protein is also an example of a NAP or CAP, wherein NAC-binding or CARD-X-binding activity can be activated under the appropriate conditions (i.e., phosphorylation, proteolysis, protein binding, pH change, and the like). For convenience of discussion, the terms "NAP" and "CAP", as used herein, are intended to include a NAP or CAP, respectively, and active fragments thereof.

Since CARD-containing proteins are commonly involved in apoptosis, the association of a NAP or CAP with NAC or CARD-X can affect the level of apoptosis in a cell. The identification by use of the methods described herein of various NAPs or CAPs can provide the necessary insight into cell death or signal transduction pathways controlled by NAC or CARD-X, allowing for the development of assays that are useful for identifying agents that effectively alter the association of a NAP with NAC or a CAP with CARD-X. Such agents can be useful, for example, for providing effective therapy for a cancer in a subject or for treating an autoimmune disease. These same assays can be used for identification of agents that modulate

the self-association of NAC via its CARD domain, NB-ARC domain, or other domains within this protein; and, they can be used for identification of agents that modulate the self-association of CARD-X with itself via its CARD domain or other domains found within this protein.

In a normal cell, a steady state level of association of NAP and NAC proteins likely occurs. This steady state level of association of NAP and NAC proteins in a particular cell type can determine the normal level of apoptosis in that cell type. An increase or decrease in the steady state level of association of NAP and NAC proteins in a cell can result in an increased or decreased level of apoptosis in the cell, which can result in a pathology in a subject. The normal association of NAP and NAC proteins in a cell can be altered due, for example, to the expression in the cell of a variant NAP or NAC protein, respectively, either of which can compete with the normal binding function of NAC and, therefore, can decrease the association of NAP and NAC proteins in a cell. The term "variant" is used generally herein to mean a protein that is different from the NAP or NAC protein that normally is found in a particular cell type. In addition, the normal association of NAP and NAC proteins in a cell can be increased or decreased due, for example, to contact of the cell with an agent such as a drug that can effectively alter the association of NAP and NAC proteins in a cell.

NB-ARC and CARD domain proteins of the invention, NACβ, NACγ and NACδ, were characterized, for example, using an *in vitro* binding assay and CARD-containing proteins were further characterized using the yeast two hybrid system. An *in vivo* transcription activation assay

such as the yeast two hybrid system is particularly useful for identifying and manipulating the association of proteins. In addition, the results observed in such an assay likely mirror the events that naturally occur in a cell. Thus, the results obtained in such an *in vivo* assay can be predictive of results that can occur in a cell in a subject such as a human subject.

A transcription activation assay such as the yeast two hybrid system is based on the modular nature of transcription factors, which consist of functionally separable DNA-binding and trans-activation domains. When expressed as separate proteins, these two domains fail to mediate gene transcription. However, transcription activation activity can be restored if the DNA-binding domain and the trans-activation domain are bridged together due, for example, to the association of two proteins. The DNA-binding domain and trans-activation domain can be bridged, for example, by expressing the DNA-binding domain and trans-activation domain as fusion proteins (hybrids), provided that the proteins that are fused to the domains can associate with each other. The non-covalent bridging of the two hybrids brings the DNA-binding and trans-activation domains together and creates a transcriptionally competent complex. The association of the proteins is determined by observing transcriptional activation of a reporter gene (see Example I).

The yeast two hybrid systems exemplified herein use various strains of S. cerevisiae as host cells for vectors that express the hybrid proteins. A transcription activation assay also can be performed using, for example, mammalian cells. However, the yeast two hybrid system is particularly useful due to the ease

of working with yeast and the speed with which the assay can be performed. For example, yeast host cells containing a lacZ reporter gene linked to a LexA operator sequence were used to demonstrate that the $CARD_L$ domain of NAC (amino acid residues 1128-1473 of SEQ ID NO:2) can interact with several CARD-containing proteins (see Examples). For example, in one case the DNA-binding domain consisted of the LexA DNA-binding domain, which binds the LexA promoter, fused to the $CARD_L$ domain of NAC and the trans-activation domain consisted of the B42 acidic region separately fused to several cDNA sequences which encoded CARD-containing proteins. When the LexA domain was non-covalently bridged to a trans-activation domain fused to a CARD-containing protein, the association activated transcription of the reporter gene.

A NAP, for example, a CARD-containing protein or an NB-ARC-containing protein also can be identified using an *in vitro* assay such as an assay utilizing, for example, a glutathione-S-transferase (GST) fusion protein as described in the Examples. Such an *in vitro* assay provides a simple, rapid and inexpensive method for identifying and isolating a NAP. Such an *in vitro* assay is particularly useful in confirming results obtained *in vivo* and can be used to characterize specific binding domains of a NAP. For example, a GST/$CARD_L$ fusion protein can be expressed and can be purified by binding to an affinity matrix containing immobilized glutathione. If desired, a sample that can contains a CARD-containing protein or active fragments of a CARD-containing protein can be passed over an affinity column containing bound GST/$CARD_L$ and a CARD-containing protein that binds to $CARD_L$ can be obtained. In addition, GST/$CARD_L$ can be used to screen a cDNA expression library, wherein binding of

the GST/$CARD_L$ fusion protein to a clone indicates that the clone contains a cDNA encoding a CARD-containing protein.

In another embodiment of the invention, methods are provided for monitoring the progress of treatment for a pathology that is characterized by an increased or decreased level of apoptosis in a cell, which methods are useful to ascertain the feasability of such treatment. Monitoring such a therapy, such as, e.g., a therapy that alters association of a NAC with a NAC-associated protein in a cell using an effective agent, can allow for modifications in the therapy to be made, including decreasing the amount of effective agent used in therapy, increasing the amount of effective agent, or using a different effective agent. In general, a test sample can be obtained from a subject having a pathology characterized by increased or decreased apoptosis, which sample can be compared to a control sample from a normal subject to determine whether a cell in the test sample has, for example, increased or decreased expression of NAC. Preferably, this control sample is a previous sample from the same patient, thereby providing a direct comparison of changes to the pathology as a result of the therapy. The level of a NAC in a cell can be determined by contacting a sample with a reagent such as an anti-NAC antibody or a NAC-associated protein, either of which can specifically bind a NAC. For example, the level of a NAC in a cell can determined by well known immunoassay or immunohistochemical methods using an anti-NAC antibody (see, for example, Reed et al., supra, 1992; see, also, Harlow and Lane, supra, (1988)).

In accordance with another embodiment of the invention, there are provided methods for determining a prognosis of disease free or overall survival in a

patient suffering from cancer. For example, it is contemplated herein that abnormal levels of NAC proteins (either higher or lower) in primary tumor tissue show a high correlation with either increased or decreased tumor recurrence or spread, and therefore indicates the likelihood of disease free or overall survival. Thus, the present invention advantageously provides a significant advancement in cancer management because early identification of patients at risk for tumor recurrence or spread will permit aggressive early treatment with significantly enhanced potential for survival. Also provided are methods for predicting the risk of tumor recurrence or spread in an individual having a cancer tumor; methods for screening a cancer patient to determine the risk of tumor metastasis; and methods for determining the proper course of treatment for a patient suffering from cancer. These methods are carried out by collecting a sample from a patient and comparing the level of NAC expression in the patient to the level of expression in a control or to a reference level of NAC expression as defined by patient population sampling, tissue culture analysis, or any other method known for determining reference levels for determination of disease prognosis. The level of NAC expression in the patient is then classified as higher than the reference level or lower than the reference level, wherein the prognosis of survival or tumor recurrence is different for patients with higher levels than the prognosis for patients with lower levels.

All U.S. patents and all publications mentioned herein are incorporated in their entirety by reference thereto. The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

1.0 *cDNA Cloning.* Jurkat total RNA was reverse-transcribed to complementary DNAs using MMLV reverse transcriptase (Stratagene) and random hexanucleotide primers. Three overlapping cDNA fragments of NAC were amplified from the Jurkat complementary DNAs with Turbo *Pfu* DNA polymerase (Stratagene) using the following oligonucleotide primer sets: primer set 1; 5'-CCGAATTCACCATGGCTGGCGGAGCCTGGGGC-3' (forward; SEQ ID NO:13) and 5'-CCGCTCGAGTCAACAGAGGGTTGTGGTGGTCTTG-3' (reverse; SEQ ID NO:14), primer set 2; 5'-CCCGAATTCGAACCTCGCATAGTCATACTGC-3' (forward; SEQ ID NO:15) and 5'-GTCCCACAACAGAATTCAATCTCAACGGTC-3' (reverse; SEQ ID NO:16), and primer set 3; 5'-TGTGATGAGAGAAGCGGTGAC-3' (forward; SEQ ID NO:17) and 5'-CCGCTCGAGCAAAGAAGGGTCAGCCAAAGC-3' (reverse; SEQ ID NO:18). The resultant cDNA fragments were ligated into mammalian expression vector pcDNA-myc (Invitrogen, modified as described in Roy et al., EMBO J. 16:6914-6925 (1997)) and assembled to full-length cDNA by ligating fragments 2 and 3 at the EcoRI site to make fragment 4, and by ligating fragments 1 and 4 at the Bst X1 site, as depicted in Figure 1A. Sequencing analysis of the assembled full-length cDNA was carried out, and splice isoforms (shown as dotted and hatched regions in Figure 1B) of NAC clones were identified. The full-length NAC nucleotide and protein sequences, including two alternatively spliced regions underlined (nucleotides 2870-2959 and 3784-3915 of SEQ ID NO:1, respectively), are presented in Figure 1C. The full length nucleotide sequence of three of the isoforms is set forth in SEQ ID NOs:1, 3 and 5, corresponding to NACβ, NACγ and NACδ, respectively.

Comparison of NAC to known protein sequences using Clustal multiple sequence alignment (Thompson et al., Nucleic Acids Research 22:4673-4680 (1994)) revealed that the CARD domain of NAC (see, e.g., residues 1373 to 1473 of SEQ ID NO:2) is similar to numerous CARD domain proteins. Further sequence analysis predicted an $\alpha_8\beta_8$ (TIM)-Barrel-like domain similar to those observed in aldolase and RuBisCo in NAC, located on the immediate amino terminal side of the predicted CARD domain (see, e.g., residues 1079 to 1364 of SEQ ID NO:2). Additionally, a portion of NAC was found to have sequence portions homologous to NB-ARC domains (see, e.g., residues 329 to 547 of SEQ ID NO:2) and a leucine-rich repeat region (see, e.g., residues 808 to 947 of SEQ ID NO:2). Based on its homology to the above proteins the protein of the invention has been termed a NAC protein, as it is a NB-ARC and CARD domain containing protein. ClustalW multiple sequence alignment with other NB-ARC and CARD domain containing proteins confirmed the homology of NAC to other proteins in both the NB-ARC region (particularly in the P-loop, or Walker A, and Walker B portions) and CARD region (Figure 1D and Figure 1E, respectively). This sequence analysis represents the first time a domain resembling a TIM-barrel domain has been identified in a protein that also contains a CARD domain, and also the first time a domain resembling a TIM-barrel domain has been identified in a protein that also contains an NB-ARC domain.

2.0 *Plasmid Constructions*. Complementary DNA encoding the CARD domain of NAC was amplified from Jurkat cDNAs with Turbo *Pfu* DNA polymerase (Stratagene) and primer set 3 as described above. The resultant PCR fragments were digested with *EcoRI* and *Xho I* restriction enzymes and ligated into pGEX-4T1 (Pharmacia) and pcDNA-myc vectors.

This region of NAC contains two alternatively spliced isoforms, termed $CARD_L$ (amino acid residues 1128-1473 of SEQ ID NO:2) and $CARD_S$ (amino acid residues 1128-1261 and 1306-1473 of SEQ ID NO:2). The region of cDNA encoding NB-ARC domain was PCR-amplified using primers SEQ ID NO:15 (forward) and SEQ ID NO:14 (reverse). The resultant PCR fragment was digested with EcoRI and Xho I restriction enzymes (yielding a fragment encoding amino acid residues 326-551 of SEQ ID NO:2) and ligated into a pGEX-4T1 and pcDNA-myc vectors.

3.0 *In vitro Protein Binding Assays*. NB-ARC, $CARD_L$, and $CARD_S$ in pGEX-4T1 were expressed in XL-1 blue *E. coli* cells (Stratagene), and affinity-purified using glutathione (GSH)-sepharose according to known methods, such as those in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley and Sons (1999). For GST pull-down assays, purified $CARD_L$ and $CARD_S$ GST fusion proteins and GST alone (0.1-0.5 µg immobilized on 10-15 µl GSH-sepharose beads) were incubated with 1 mg/ml of BSA in 100 µl Co-IP buffer [142.4 mM KCl, 5mM M_gCl_2, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.2% NP-40, 1 mM DTT, and 1 mM PMSF] for 30 min. at room temperature. The beads were then incubated with 1 µl of rat reticulocyte lysates (TnT-lysate; Promega, Inc.) containing ^{35}S-labeled, *in vitro* translated $CARD_L$, $CARD_S$, or control protein Skp-1 in 100 µl Co-IP buffer supplemented with 0.5 mg/ml BSA for overnight at 4°C. The beads were washed four times in 500 µl Co-IP buffer, followed by boiling in 20 µl Laemmli-SDS sample buffer. The eluted proteins were analyzed by SDS-PAGE. The bands of SDS-PAGE gels were detected by fluorography.

The resultant homodimerization pattern reveals that $CARD_L$-$CARD_L$, $CARD_S$-$CARD_S$, and both $CARD_L$-$CARD_S$ containing

lanes have very strong signals, whereas lanes containing control GST alone and control Skp-1 have negligible signals (Figure 2A). Thus, CARD domains of the invention NAC show a very strong ability to self-associate *in vitro*.

In vitro translated Apaf-1(lacking its WD domain), CED4, and control Skp-1 proteins were subjected to GST pull-down assay using GSH-sepharose beads conjugated with GST, GST-$CARD_L$, and GST-$CARD_S$ as described above. Both lanes containing GST-$CARD_S$ and lanes containing GST-$CARD_L$ yielded very strong signals when incubated with either Apaf-1(-WD) or CED4, whereas, the controls GST alone and Skp-1 again yielded negligible signals (Figure 2B). Thus, in addition to self-association, CARD domains of the invention NAC demonstrate the ability to *in vitro* associate with other CARD-containing proteins.

4.0 *Protein Interaction Studies in Yeast*. EGY48 yeast cells (*Saccharomyces cerevisiae*: MATα, trpl, ura3, his, leu2::plexApo6-leu2) were transformed with pGilda-CARDL plasmids (his marker) encoding the LexA DNA binding domain fused to: CARD domains of NAC ($CARD_L$) and caspase-9; pro-caspase-8; Apaf-1 without its WD domain; Bcl-XL, Bax and Bcl-2 without transmembrane domains. EGY48 were also transformed with vector pJG4-5 (trpl marker) encoding the above listed group of proteins and additionally vRas and FADD as target proteins, fused to B42 transactivation domain, and the cells were transformed with a LexA-LacZ reporter plasmid pSH1840 (ura3 marker,), as previously described (Durfee et al., 1993; Sato et al., 1995). Sources for cells and plasmids were described previously in U.S. Patent 5,632,994, and in Zervous et al., Cell 72:223-232 (1993); Gyuris et al., Cell 75:791-803 (1993); Golemis et al., In Current

Protocols in Molecular Biology (ed. Ausubel et al.; Green Publ.; NY 1994), each of which is incorporated herein by reference. Transformants were replica-plated on Burkholder's minimal medium (BMM) plates supplemented with leucine and 2% glucose as previously described (Sato et al., Gene 140:291-292 (1994)). Protein-protein interactions were scored by growth of transformants on leucine deficient BMM plates containing 2% galactose and 1% raffinose.

Protein-protein interactions were also evaluated using β-galactosidase activity assays. Colonies grown on BMM/Leu/Glucose plates were filter-lifted onto nitrocellulose membranes, and incubated over-night on BMM/Leu/galactose plates. Yeast cells were lysed by soaking filters in liquid nitrogen and thawing at room temperature. β-galactosidase activity was measured by incubating the filter in 3.2 ml Z buffer (60 mM, Na_2HPO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$) supplemented with 50 µl X-gal solution (20mg/ml). Levels of β-galactosidase activity were scaled according to the intensity of blue color generated for each transformant.

The results of this experiment showed colonies on leucine deficient plates for yeast containing NAC-$CARD_L$/LexA fusions together with caspase-9/B42, Apaf-1/B42, and Bax/B42 fusions (Figure 3). In addition, the NAC-$CARD_L$/LexA:caspase-9/B42 and NAC-$CARD_L$/LexA:Apaf-1/B42 cells had significant amounts of LacZ activity. The cells containing the complementary fusions caspase-9/LexA:NAC-$CARD_L$/B42 and Apaf-1/LexA:NAC-$CARD_L$/B42 also grew on leucine deficient plates and showed significant LacZ activity. Thus all four indicators of protein:protein interaction confirmed that the $CARD_L$ domain of NAC interacts with the CARD

domains of caspase-9 and with Apaf-1. Partial indication of the protein:protein interactions with NAC-$CARD_L$ were observed for Bax, caspase-8, Bcl-XL and Bcl-2, suggesting that a broad range of CARD domain proteins also interact with the CARD domain of NAC.

Similar two-hybrid interaction experiments have been performed using the CARD domain of the CARD-X protein. Table I summarizes the results of the two-hybrid experiments wherein a fusion protein containing the DNA-binding domain of the LexA protein expressed from the pGilda plasmid and a CARD domain from CARD-X or several other CARD-containing proteins, including CARDIAK, NAC ($CARD_L$), Apaf-1, caspases-2, 9, and 11, were expressed in the sames cells as CARD domains from CARD-X, CARDIAK, NAC($CARD_L$), caspase-9 and cIAP-2, expressed as fusion proteins with a transactivation domain from the B42 protein from the pJG4-5 plasmid, as described above. As shown, the CARD domain of CARD-X interacted with itself but not with the CARD domains of other proteins.

TABLE I

Yeast Two Hybrid Analysis of CARD-X:CARD interactions

	pGilda	pJG4-5	Results
1	CARD-X CARD	CARD-X-CARD	+++
2	CARD-X CARD	CARDIAK	-
3	CARD-X CARD	NAC-$CARD_L$	-
4	CARD-X CARD	Caspase-9 CARD	-
5	CARD-X CARD	cIAP-2	-
6	CARDIAK	CARD-X CARD	-
7	NAC-$CARD_L$	CARD-X CARD	-
8	APAF C3+C4	CARD-X CARD	-
9	Caspase-2	CARD-X CARD	-
10	Caspase-11	CARD-X CARD	-
11	Caspase 9-C-terminus	CARD-X CARD	-
12	CARDIAK	CARDIAK	++++

5.0 *Self-Association of NB-ARC domain of NAC. In vitro* translated, ^{35}S-labeled rat reticulocyte lysates (1 µl) containing NB-ARC or Skp-1 (used as a control) were incubated with GSH-sepharose beads conjugated with purified GST-NB-ARC or GST alone for GST pull-down assay, resolved on SDS-PAGE and visualized by fluorography as described above. One tenth of input were loaded for NB-ARC or Skp-1 as controls. In this assay, the NB-ARC-containing fragment of NAC demonstrates a strong ability to homodimerize (Figure 4).

The ability to self-associate and to bind other known CARD domains establishes the CARD domains of NAC,

$CARD_S$ and $CARD_L$, as capable of the same protein-protein interactions observed in other known CARD domains. The ability of CARD-X to self-associate also establishes this protein as having the same protein-protein interaction properties of known CARD proteins. Thus two isoforms of a new human CARD domain have been characterized, and a highly related sequence of another human protein CARD-X has also been characterized. In addition, the ability of the putative NB-ARC domain of NAC has been shown to both self-associate, establishing this domain as capable of the same protein-protein interactions observed in other known NB-ARC domains. Therefore, the NAC protein has been demonstrated to contain both a functional CARD domain and a functional NB-ARC domain.

6.0 Protein-Protein Interactions of NAC. Transient transfection of 293T, a human embryonic kidney fibroblast cell line, were conducted using SuperFect reagents (Qiagen) according to manufacturer's instructions. The cDNA fragments encoding full-length CED4 and the truncated form of Apaf-1 (Apaf-1ΔWD) comprising amino acids 1-420 of the human Apaf-1 protein were amplified by PCR and subcloned into pcDNA3HA at EcoRI and Xho I sites. Expression plasmids encoding catalytically inactive forms of pro-Casp8 [pro-Casp8 (C/A)] was prepared by replacing Cys 377 with an Ala using site-directed mutagenesis and pro-Casp9 [pro-Casp9 (C/A)] has been described previously, Cardone et al., Science 282:1318-1321 (1998)). 293T cells were transiently transfected with an expression plasmid (2 μg) encoding HA-tagged human Apaf-1ΔWD, CED4, pro-Casp8 (C/A) or C-Terminal Flag-tagged pro-Casp9 (C/A) in the presence or absence of a plasmid (2 μg) encoding myc-tagged NAC (encoding amino acid residues 1-1261 and 1306-1473 of SEQ ID NO:2). After 24 hr growth in culture, transfected cells were collected

and lysed in Co-IP buffer [142.4 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.1 % NP-40, and 1 mM DTT] supplemented with 12.5 mM β-glycerolphosphate, 2 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1X protenase inhibitor mix (Boehringer Mannheim). Cell lysates were clarified by microcentrifugation and subjected to immunoprecipitation using either a mouse monoclonal antibody to myc (Santa Cruz Biotechnologies, Inc) or a control mouse IgG. Proteins from the immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblot analysis using anti-HA antibodies followed by anti-myc antibodies using a standard Western blotting procedure and ECL reagents from Amersham-Pharmacia Biotechnologies, Inc. (Krajewski et al., Proc. Natl. Acad. Sci. USA 96:5752-5757 (1999)).

The results show that NAC of the invention interacts with other NB-ARC and CARD-containing proteins, Apaf-1 (Figure 5A) and CED-4 (Figure 5B), and additionally with caspase-8 (Figure 6A), but not with caspase-9 (Figure 6B). This is in contrast with the observed interaction between caspase-9 and the $CARD_L$ domain of NAC from the above described yeast two-hybrid assay. This may be due to the regulation of the full-length NAC in terms of its ability to interact with pro-caspase-9 such that NAC is in either a latent (off) or active (on) conformation, analogous to Apaf-1 which binds pro-caspase-9 only when cytochrome c is produced to induce a conformational change in Apaf-1. As with NAC, if only the CARD domain of Apaf-1 is expressed, it will bind to pro-caspase-9 independently of the coactivator, cytochrome c (Qin et al., Nature 399:549-557 (1999)).

Although the invention has been described with reference to the examples above, it should be understood

that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.